The role of immediate early transcription factors in the regulation of peripheral circadian clocks

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The role of immediate early transcription factors in the regulation of peripheral circadian clocks

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RÉSUMÉ

Les organismes synchronisent leurs comportements, leur physiologie et leur métabolisme avec l'environnement qui, dû à la rotation de la terre autour de son propre axe, change périodiquement. A cette fin ils utilisent des horloges circadiennes, qui fournissent des mécanismes pour la synchronisation interne. Dans le système des mammifères, l'horloge centrale se trouve dans le noyau suprachiasmatique du cerveau (SCN). La phase de celle-ci est entraînée directement par les cycles de luminosité-obscurité, tandis que la phase des organes périphériques, comme le foie, sont entraînées par des signaux systémiques contrôlés directement ou indirectement par le SCN. Celui-ci emet des signaux rhythmiques, comme la température du corps ou des facteurs saguins qui peuvent établir la cohérence des phases dans l'animal. Cependant, les mécanismes de synchronisation sont encore mal compris pour les organes périphériques. On a préalablement observé qu'un choc de sérum peut synchroniser les horloges des cellules en culture. Ce traitement provoque l'activation de plusieurs facteurs de transcription précoces immédiats (IETFs), comme c-Fos, PER1 et PER2. L'hypothèse de travail dans notre laboratoire est donc que les facteurs IETFs participent à la synchronisation des horloges périphériques.

Une méthode appelée STAR-PROM (Synthetic TAndem Repeat PROMoter screening) a été établie dans notre laboratoire, afin d'identifier des facteurs IETFs qui répondent aux facteurs du sang. Dans cette méthode l'activation de facteurs IETF se manifeste par l'augmentation de l'activité de la luciférase et sert ainsi de témoin lumineux. Les principes de base de STAR-PROM sont 1) la fréquence élevée des sites de liaison pour des facteurs de transcription se liant à l'ADN dans une séquence d'ADN synthétique aléatoire, et 2) une induction robuste de témoin lumineux, lorsque les sites de liaison pour les facteurs de transcription sont mis en tandem en multiples répétitions (Gerber et al.). La bibliothèque contenait ~ 2800 clones avec 7 répétitions en tandem de séquences aléatoires en amont d'un promoteur minimal et un gène rapporteur de la luciférase de courte durée dans un vecteur lentiviral. Au total, cette bibliothèque permet d'échantillonner environ 400'000 paires de bases d'ADN aléatoire pour la détection de sites de liaison non-palindromiques et environ 200'000 paires de bases pour la présence de sites de liaison palindromiques (les deux brins de l'ADN possède une séquence différente pour les sites non-palindromiques). Cette

complexité de séquence devrait nous permettre de trouver au moins un site de liaison pour la plupart des facteurs de transcription. Un protocole de production et de criblage à haut débit par des vecteurs lentiviraux a été développé. Contrairement à l'étude précédente, ce projet a été réalisé en utilisant des cellules HepaRG, une lignée de cellules de foie humain, avec des échantillons de plasma humain. Cette étude a révélé 30 clones qui ont été induits par le sérum ou le plasma sanguin. Tous ces clones ont été vérifiés comme des vrais positifs dans des expériences ultérieures. Les propriétés de deux clones positifs, avec des sites de liaison pour les facteurs de transcription FOXA2 et le récepteur nucléaire orphelin NR4A1, ont été étudiées plus précisément,.

Il a été constaté que l'amplitude de l'induction de FOXA2 par le sérum de rat ou le plasma sanguin humain suivait un rythme circadien. D'autre part les résultats obtenus dans les approches pharmacologiques ont suggéré que Ca^{2+}, MEK1 / 2 kinase, et tyrosine kinase participent aux processus de transduction de signaux par les facteurs sanguins. Une expérience de digestion avec protéase a indiqué que ces derniers sont de nature protéique. Nous avons noté avec intérêt que la thrombine, un des agents de la coagulation du sang, peut induire les activités de FOXA2, de SRF, et des facteurs de transcription pas encore identifiés. De plus, la thrombine peut synchroniser l'horloge circadienne *in vitro*, probablement via l'activation de *Per2*. Un rôle de FOXA2 dans la régulation de l'horloge circadienne a été confirmé par des expériences de perte de fonction dans les cellules HepaRG et dans le foie de souris. In vitro, l'effet de diminution de FOXA2 a provoqué un amortissement conséquent de l'amplitude de l'horloge circadienne. En concordance avec ces observations faites *in vitro*, l'enregistrement de la bioluminescence *in vivo* a révélé que l'absence de FOXA2 dans le foie de souris a provoqué une diminution progressive de l'expression du rapporteur circadien Rev-Erbα-luciférase. La diminution de l'expression du gène *Rev-Erba* endogène en absence de FOXA2 a été confirmée par la détermination de l'accumulation de l'ARNm *Rev-Erba* dans le foie de type sauvage et de souris knockout pour *FoxA2*.

Le récepteur nucléaire orphelin NR4A1 montre une expression circadienne dans le foie des souris et une liaison circadienne à l'ADN dans des expériences EMSA (electrophoretic mobility shift assays) avec des extraits nucléaires de foie préparés à partir de souris sacrifiées à des intervalles de 4 heures pendant 24 heures. Pour

comprendre comment NR4A1 régule le système circadien, l'effet de l'activation de NR4A1 par son agoniste synthétique cytosporone B (CSNB) a été analysé sur les témoins de gènes circadiens. CSNB diminue l'expression de témoin Bmal1-luciférase, mais pas celle du rapporteur *Per2*-luciférase dans les cellules HepaRG. Dans des animaux soumis à une inversion alimentaire, la phase d'expression de NR4A1 change avant celle d'autres gènes de l'horloge tels que *Rev-Erbα*. Jusqu'à présent, le ligand physiologiquement important de NR4A1 est encore inconnu. Dans des essais *in vitro* l'activité de NR4A1 peut être induite par des facteurs sériques. Par contre, l'induction est diminuée lorsque le sérum est délipidé avec des solvants organiques ou du charbon actif, ce qui suggère que l'activité de NR4A1 est stimulée par des lipides présents dans le sérum. La caractérisation des lipides sériques sera poursuivie dans des expériences futures.

En conclusion, deux nouveaux mécanismes ont été identifiés qui montrent la façon dont les signaux véhiculés par le sang peuvent réguler l’expression des gènes circadiens: 1) L’activation de FOXA2 par des protéines, y compris la thrombine. Ce processus pourrait impliquer les récepteurs transmembranaires PAR 2) L’activation du récepteur nucléaire orphelin NR4A1 par des lipides.

ABSTRACT

Organisms synchronize their behavior, physiology, and metabolism with the periodically changing environmental condition using internal timing mechanisms or circadian clocks. In mammalian system, the master clock in the brain's suprachiasmatic nucleus (SCN), is entrained directly by light-dark cycles, while the peripheral organs like liver are entrained by systemic cues directly or indirectly controlled by the SCN, like body temperature or blood-borne signals. However, the mechanisms of phase-entrainment in peripheral organs are still poorly understood. Based on the observation that the up-regulation of immediate early transcription factors (IETFs), like c-FOS, PER1, and PER2 were observed after serum shock in cultured cells, the working hypothesis in our laboratory is that IETFs participate in peripheral clock synchronization.

A method called STAR-PROM (Synthetic Tandem Repeat Promoter) screening previously was established in our laboratory to identify IETFs responding to blood-borne factors, using luciferase activity as a readout. The underlying principles of STAR-PROM are 1) the high frequency of transcription factor binding sites in random synthetic DNA and 2) the robust reporter induction when transcription factors binding sites are put in tandem repeats (Gerber et al.). The library contains ~2,800 clones with 7-copy tandem repeats of random sequences upstream of a minimal promoter and the reporter gene encoding a short-lived luciferase in a lentiviral vector. Altogether, this library is expected to sample about 400,000 bp of random DNA for the presence of non-palindromic transcription factor binding sites and about 200,000 base pairs for the presence of palindromic binding sites (the two strand of DNA have a different sequence for non-palindromic sites). This sequence complexity should allow us to find at least one binding site for most transcription factors. A high-throughput lentivirus production and screening protocol was developed. In contrast to the previous study, in this project the screen was performed using HepaRG cells, a human liver cell line, with human plasma samples. In the primary screen, 30 clones were serum/plasma responsive. All of these clones were verified as true positives in subsequent experiments. Two of the positive clones were further characterized. They contain binding sites of the transcription factor FOXA2 and the orphan nuclear receptor NR4A1.

The induction of FOXA2 by rat or human plasma showed a diurnal pattern. The results obtained in pharmacological approaches suggested that Ca^{2+}, MEK1/2 kinase, tyrosin kinases were involved in the signaling transduction process from the blood-borne signals to FOXA2. Protease digestion experiment suggested that the blood-borne factors are of proteinaceous nature. Interestingly, the blood clotting agent thrombin can induce the reporters for FOXA2, SRF, and unidentified transcription factors stimulating the expression of other positive clones found in the screen. Moreover, thrombin can synchronize circadian clocks *in vitro*, probably via the activation of *Per2*. The role of FOXA2 in the circadian clock regulation was further examined by depletion of FOXA2 in HepaRG cells and in mouse liver. *In vitro*, the knockdown of FOXA2 causes a rapid dampening of the circadian clock. *In vivo* bioluminescence recording revealed that the knockout of FOXA2 in mouse liver caused a gradual decrease in the expression of the circadian reporter *Rev-Erba*-luciferase. The down-regulation of the endogenous clock genes was confirmed by determining mRNA accumulation in the livers of wild-type and *FoxA2* knockout mice.

The orphan nuclear receptor NR4A1 shows circadian expression in mouse liver and a circadian DNA binding pattern in electrophoretic mobility shift assay (EMSA) with liver nuclear extracts prepared from mice sacrificed at 4-hour intervals around the clock. To understand how NR4A1 regulates the circadian system, the effect of activation of NR4A1 by its synthetic agonist cytosporone B (CsnB) on circadian gene reporters was examined. CsnB down-regulates *Bmal1*-luciferase reporter gene expression, but not *Per2*-luciferase reporter gene expression in HepaRG cells. *In vivo*, under food inversion condition, the expression of NR4A1 changes prior to that of other clock genes, such as *Rev-Erba*. Until now, the physiological ligand of NR4A1 is still unknown. In the cell-based reporter assay, NR4A1 activity can be induced by serum factors. The induction is diminished when the serum is delipidated with organic solvents or activated charcoal, suggesting that NR4A1 activity is stimulated by lipid(s) present in the serum. The characterization of the serum lipids will be continued in future experiments.

In conclusion, two new mechanisms were identified for how blood-borne signals may regulation circadian gene expression: 1) signaling by proteins, including thrombin, through, FOXA2, perhaps involving PAR transmembrane receptors, and 2) signaling by lipids via the orphan nuclear receptor NR4A1.

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1. Introduction

1.1 Biological clocks and health

"To every thing there is a season, and a time to every purpose under the heaven: A time to be born, and a time to die; a time to plant, and a time to pluck up that which is planted." The book of Ecclesiastes beautifully documented the seasonal changes of the life cycles on earth already thousands of years ago. As the Earth rotates on a tilted axis, there are different lengths of the day and different seasonal changes depending on the location on this planet.

Plant leaf movements are rhythmic to maximize the efficiency of photosynthesis. Nocturnal animals such as rats can avoid predators that are active during the day. Therefore, the emergence of biological clocks provided selective advantages for organisms because different species can live in the same place, but can temporally occupy different niches. In mammals, the physiology is highly dependent on the time of the day, for example, insulin secretion, urine production, body temperature, fat accumulation, inflammation, etc. (Bass, 2012). With modern day technology, genome-wide and high-throughput studies revealed that diurnal changes occur almost everywhere inside our body. For example, a recent transcriptome study in mice livers showed about 16% of the mRNA had rhythmic accumulation (Du et al., 2014). A proteomic study around the clock done by Reddy and colleagues revealed about 20% of the soluble proteins displayed circadian accumulation in abundance in the liver (Reddy et al., 2006). Similarly, mouse liver metabolome analysis revealed that nucleotide and carbohydrate metabolites peaked at the light phase while amino acid and xenobiotic metabolism-related compounds peaked at night (Eckel-Mahan et al., 2012).

Nowadays most of the people residing in the developed world are inevitably affected by the 24-hours non-stop lifestyle. Most of us are subjected to various degrees of disruption to our biological clock. For example, the natural rhythm of shift workers is significantly perturbed, as they are exposed to long hours of artificial light and non-natural feeding schedules. Every one of us is slightly affected in our everyday life. For example, some trivial personal habits like checking email or playing around with one's cell phone before bedtime, or a cup of espresso in the evening can disrupt one's normal internal rhythm. The light from computers and cell

phones or caffeine can directly impact on the biological clock (Burke et al., 2015). Disruption of the normal daily rhythm has an enormous impact on public health, including an increased risk for cancer, cardiovascular disease, obesity and diabetes (Paschos et al., 2012; Marcheva et al., 2010; Mattson et al., 2014). One emerging theme is that the time of food intake is essential for our health. In the rat night-work model, the rhythmic blood glucose level and locomotor activity were flattened, and the rats were more prone to obesity (Salgado-Delgado et al., 2010). A study from Turek's group showed that in mice the intake of high-fat diet during the 12-light phase caused a higher weight gain compared to mice fed during the 12-dark phase (Arble et al., 2009). Another similar study by Panda's group showed mice fed with high-fat diet in a restricted time were protected against obesity-related diseases, hyperinsulinemia, hepatic steatosis and inflammation when compared to mice fed *ad libitum* (Hatori et al., 2012). These studies provided useful information on how we can prevent obesity by changing one's eating schedules. Researchers developed a smart phone application to track the feeding behaviors of volunteers and gave suggestions on their feeding time schedules. After 16 weeks of the experimental period, the subjects were able to reduce weight gain and had better sleep quality (Gill and Panda, 2015).

1.2 Molecular components of the circadian clock

The term 'circadian' is derived from the Latin words "circa diem" meaning 'approximately one day'. Different parameters are used to describe circadian oscillators. The oscillation can be graphically represented as a sine wave curve. The highest point is the peak, and the lowest point is the trough. Period length is the time needed to complete one cycle of the oscillation. The amplitude is half the distance from the peak to the trough. To describe the dynamic changes of the behavior of the oscillator, we will need a reference point for comparison. The phase of the oscillator describes the position of a particular point in the oscillator, for example, the peak or the trough. Phase shift means the change of the phase of the oscillator, while period length and amplitude are not affected. Figure 1.1 is the graphic representation of the terms discussed above.

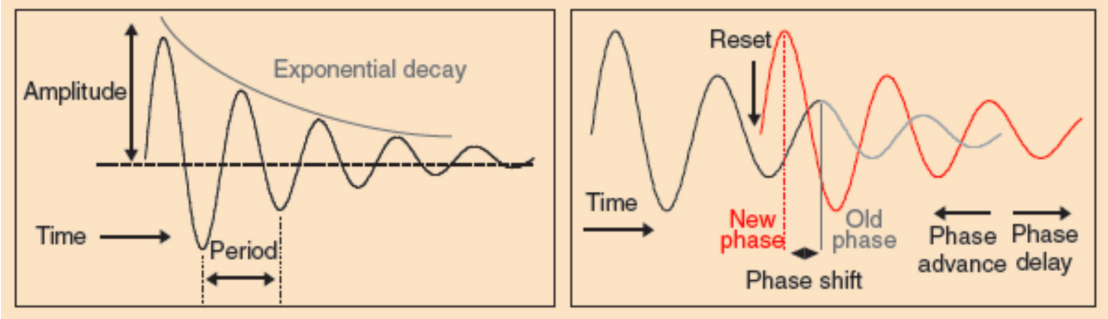

Figure 1.1 Terms used to describe oscillations (adapted from Liu et al., 2007). The gene expression level was plotted as a function of time. If the gene expression oscillates over time, a sine wave curve can be observed. In the graph is shown an exponential decay of gene expression over time as observed *in vitro* experiments. The amplitude is the distance from the peak to the mid-point of the expression. The period is shown as the time between two peaks or trough. Usually, the peak or the trough is used as a reference point when describing the shift of an oscillation, which is also called *phase shift*. In the graph, after the reset of the oscillation, the peak of the red curve (new phase) comes earlier then the peak of the original curve (old phase), which is called a phase advance. If the peak of the red curve comes later then the original curve, it is called a phase delay.

There are three criteria for a biological oscillation to be called circadian. First, the time to complete one cycle, i.e. the period length, should be approximately 24 hours under free-running conditions. This means the oscillation must be generated without any external input. Second, the oscillator(s) should be entrainable, meaning the oscillator(s) can be adjusted by external timing cues. This criterion also suggests that biological oscillators are adaptive to different environmental changes. These external timing cues are called *Zeitgebers*, a German word meaning “time giver” or “synchronizer”, for example, the daily light/dark cycle. Third, the oscillator should be temperature-compensated, meaning the period length should remain approximately 24-hours regardless of changes in the temperature within the physiological range. Since temperature would affect the kinetics of biochemical processes inside an organism, to keep time for the organism, the endogenous clock should have a mechanism to compensate for the influence of changes of the kinetics in the oscillator. However, temperature itself can be a cue for entrainment under certain circumstances (Brown et al., 2002; Buhr et al., 2010; Saini et al 2012).

Circadian cycles are found in most organisms, including cyanobacteria, fungi, insects, and mammals. However, the regulatory mechanisms of the circadian clock across species are different, suggesting independence in the evolution of the various clockworks. Even though the mechanisms seem not to be conserved among species, the underlying logic can be generalized to rely on intertwined feedback loops. The simplest oscillation model is shown in Figure 1.2. Let's consider the following regulatory relationship. A (activator) actives R (repressor) and R inhibits A. Because of the activation by A, the quantity and activity of R accumulates over a certain threshold, and R starts to inhibit A. Due to the inhibition of A, the activity of A decreases, which causes the decrease of activation of R. Thus, after some time, as the amount of R decreases, the inhibition on A is relieved, so that a new cycle of activation of R by A starts. In real life the situation is even more complicated. The period length and the amplitude of the oscillation depend on the kinetics of the activation and repression, and of the half-life of the activator and repressor.

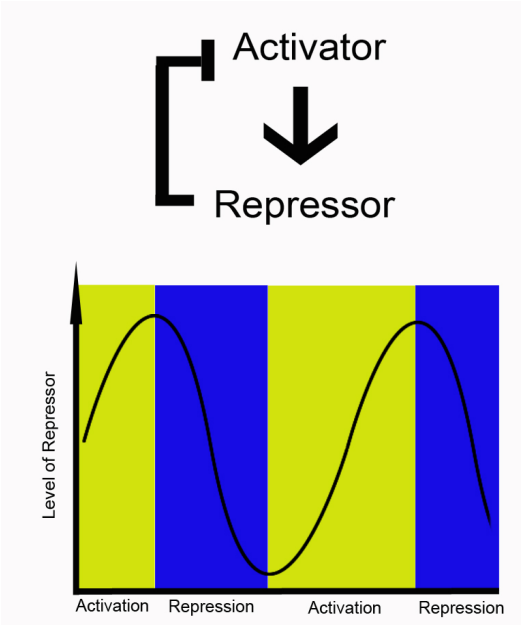

Figure 1.2 The simplest oscillator circuit. The activator drives the expression of the repressor, and the repressor inhibits its own expression after reaching a threshold. During the activation period, the level of the repressor increases. Upon reaching the threshold, the repressor inhibits its own synthesis, therefore the level of the repressor drops (repression period). Once the level of the repressor decreases, a new cycle of activation can occur. In reality, additional mechanisms are required to increase the delay between activation and repression. Without such additional delay mechanism the system would quickly fall into equilibrium.

There are reports on circadian oscillators solely operated by biochemical reactions in the absence of transcription and translation. For example, in cyanobacteria the KaiABC complex undergoes ATP hydrolysis cycles with close to 24-hours period length (Nakajima et al., 2005), and in red blood cells there is rhythmic redox activity (O'Neill and Reddy, 2011). The earliest evidence suggesting that there is a genetic basis of circadian clocks came from studies on the eye of the snail Aplysia. Transcription inhibition by the reversible drug DRB (Raju et al., 1991) and protein synthesis inhibition by anisomycin affected both the phase and period length of the circadian clock in the eye of Aplysia (Jacklet, 1977). Later, the identification of Drosophila mutants showing altered circadian activity strongly suggested a genetic basis of circadian rhythms (Konopka and Benzer, 1971). Circadian rhythms are found in almost all cell types of an organism. The more generally observed mechanisms of the circadian clock consist of the transcription-translation feedback loops (TTFL). In *Drosophila*, the genes are named after the mutant fly phenotype. The activators of the oscillators are two transcription factors, Cycle and Clock, while the repressors are Period (Per) and Timeless (Tim) (Crane and Young, 2014). In the 1990s, the cloning of the mammalian circadian clock genes revealed a similar genetic architecture as the fly circadian clocks. However, gene duplications rendered the mammalian circadian circuitry more complex (Vitaterna et al., 1994; Barnes et al., 2003; Tei et al., 1997; Zheng et al, 1999; van der Horst et al., 1999).

In the mammalian system, the TTFL consists of the bHLH-PAS transcriptional activators BMAL1 and CLOCK, the PERIOD (PER), CRYPTOCHROME (CRY) and REV-ERB proteins and the E-box (E box/E_0 box: CACGTK) (Gekakis et al., 1998; Hogenesch et al., 1997; Ueda et al., 2005; Yoo et al., 2005), and the RRE (ROR/REV-ERB binding element: WAWNTRGGTCA) (Harding and Lazar, 1993; Preitner et al., 2002; Ueda et al., 2002). BMAL1 interacts with CLOCK to form a heterodimeric activator complex, which activates the expression of repressor genes like *Per2* and *Cry* by binding to the E-box in their promoters (Yoo et al., 2005). When the repressor levels accumulate over a certain threshold, PER and CRY bind to the BMAL1/CLOCK complex and inhibit the activity of their activators. BMAL1/CLOCK also activates the transcription of *Rev-Erbα* and *Rev-Erbb*, which repress the transcription of *Bmal1*. The activation of *Bmal1* is thought to be

accomplished through the binding of ROR to the RRE in the *Bmal1* promoter (Liu et al., 2008).

DEC1/2 are two other bHLH transcription factors thought to participate in the TTFL by competition with the BMAL1/CLOCK heterodimer for binding to the E-box e.g., in the *Per1* promoter (Honma et al., 2002). BMAL1/CLOCK drives the expression of *Dec1* by binding to the E-box in the *Dec1* promoter region (Kawamoto et al., 2004). However, the knockout mutants of *Dec1/2* only show very little lengthening of the free-running period length, casting doubts on their role in the circadian clock (Rossner et al., 2008). In the suprachiasmatic nucleus, but not in peripheral tissues, NPAS2 (Neuronal PAS domain protein 2) can functionally replace CLOCK. It also forms s with BMAL1(Dioum et al., 2002; DeBruyne et al., 2007). Surprisingly, both NPAS2 and REV-ERBa are have been reported to bind heme (Raghuram et al., 2007), suggesting a regulation of both factors by molecules like CO and NO (Kaasik et al., 2004). Figure 1.3 showed a simplified model of mammalian circadian clock.

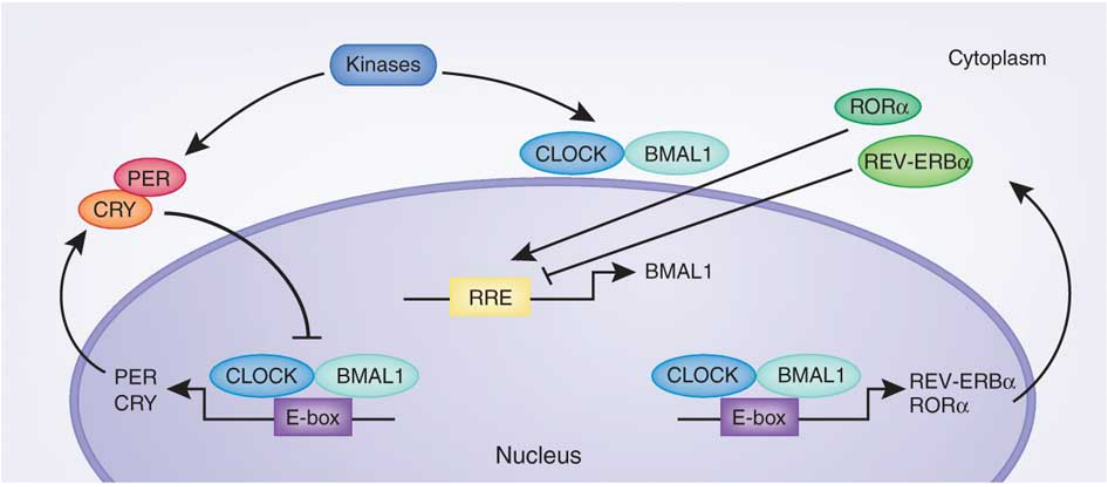

Figure 1.3 Simplified version of the mammalian circadian oscillator (adapted from Masri and Sassone-Corsi, 2010). The core loop is made out of a positive limb (BMAL and CLOCK/Npas2) activating transcription of the negative limb factors *Period* and *Cry* by binding to the E-box elements in their promoters. The accumulation of PER and CRY cause the repression of their own expression. At the same time, BMAL1/CLOCK also drives the expression of *Rev-Erbα* and ROR, which REV-ERB factors repress the expression of *Bmal1* and *Clock and RORα* activates the expression of *Bmal1*. Additional kinases regulate the stability and activities of *PER* and *BMAL1* by phosphorylation.

The rhythmic activity of transcription factors is accompanied by cyclic transitions in chromosome structure. For example, using *Dbp* as a model locus, the BMAL1/CLOCK binding and the chromatin transitions were mapped (Ripperger and Schibler, 2006). During the activation phase, histone H3K9 acetylation, H3K4 trimethylation and overall reduction of the density of histone H3 were detected. During the repression phase, H3K9 dimethylation, binding of the HP1 protein and an increase in histone density were observed. Later, genome-wide studies of histone marks also showed oscillating patterns of H3K4me3 at the promoter and H3K27ac at the enhancer (Koike et al., 2012; Vollmers et al., 2012). These histone modification changes can be explained by the recruitment of histone modifying enzymes to the chromatin by core clock components, such as CLOCK, which interacts with p300 histone acetyl transferases (Etchegaray et al., 2003) and REV-ERBα, which recruits HDAC3 and consequently represses the expression of genes (Feng et al., 2011).

The main question is still how the TTFL generates near 24-hour rhythms? According to the basic logic of the feedback loop model mentioned before, several parameters, like the binding affinity of the repressors to the activators, the rate of synthesis and degradation of the activators and the repressors would determine the turnover of the whole cycle. The 'stoichiometric balance' model proposed by Kim and Forger predicts that the optimal ratio between such activators and repressors is around 1:1 (Kim and Forger, 2012). Any imbalance of the ratio between activators and repressors would affect the period length and the robustness of circadian rhythms.

Inside the cells, the quantity and the activity of the clock proteins are carefully regulated by post-translational modification. For example, the stability and activity of PER2 are regulated by phosphorylation. Casein kinase (CK1δ/ε) phosphorylates PER2 (Maier et al., 2009) while Protein phosphatase 1 dephosphorylates PER2 (Gallego et al., 2006). In humans, mutation at the phosphorylation site 662 of PER2 is associated with familial advanced sleep phase syndrome (FASPS) (Xu et al., 2007). Interestingly, PER2 is also found to be O-GlcNAcylated. It is believed that OGT competes with the kinases to regulate the stability and activity of PER2 (Kaasik et al., 2013).

There are two different ubiquitin ligase components, FBXL3 and FBXL21 known to regulate the degradation of CRY (Yoo et al., 2013). FBXL3 is found to act strongly inside the nucleus, while FBXL21 has two different roles inside and outside of the nucleus. Inside the nucleus FBXL21 protects CRY against the degradation by FBXL3, but it promotes CRY degradation in the cytoplasm. Besides, the degradation of CRY can be regulated by external signals. For example, AMPK phosphorylates CRY1 and promotes its degradation by FBXL3 (Lamia et al., 2009). In response to an inflammation signal, TNF-α, the deubiquitination enzyme USP2a stabilizes CRY1 (Tong et al., 2012). CRY1 and CRY2 can be phosphorylated by MAPK in vitro, which decreased its ability to inhibit BMAL1 (Sanada et al., 2004). Moreover, the phosphorylation of BMAL1 at Thr-534 suppresses its transcriptional activity (Sanada et al., 2002).

In addition to the post-translational modifications, structural studies by Pratch and colleagues suggested an intrinsic property of the BMAL1 affects the period length of the circadian clock, as shown in figure 4 (Xu et al., 2015). They found that the C-terminal part of BMAL1 where CRY binds is highly unstructured. Different mutant versions in the C-terminal of BMAL1 caused the difference in the period length of the cells. They found that there is a correlation between the binding affinity of CRY to the different version of BMAL1 protein and the period length. The higher the affinity of CRY binding to BMAL1, the shorter the period length and vice versa (as illustrated in figure 1.4) (Hui and Ripperger, 2015).

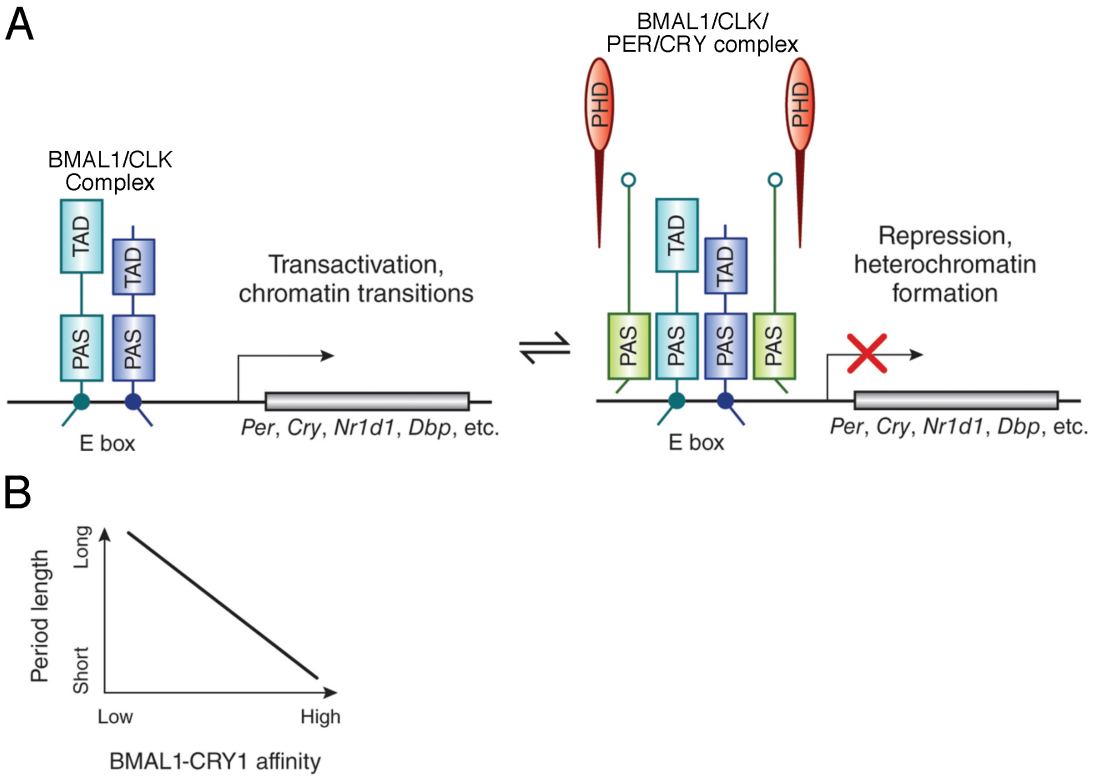

Figure 1.4 The binding affinity between activators and repressors determines the period length of the circadian oscillator (modified from Hui & Ripperger, 2015). A) The activator complex BMAL1/CLOCK drives the gene expressions of other clock components, such as *Per* and *Cry*. The repressor proteins PER and CRY form a complex that binds to the BMAL1/CLOCK heterodimer and inhibit its transactivation activity. B) Based on structural studies, mutations in C-terminal regions of BMAL1 alter the binding affinity with CRY. There is an inverse relationship between the binding affinity of BMAL1 and CRY; The higher the affinity, the shorter the period length and vice versa (Xu et al., 2015).

1.3 The signaling pathways in synchronization of the circadian clocks

Almost every cell in the body contains circadian clocks. Observations from single-cell imaging showed that self-sustained circadian rhythms exist in individual cells and that the phases of these rhythms are transmitted to the daughter cells after cell division (Nagoshi et al., 2004). As the molecular architecture of the circadian oscillators is based on TTFL and transcription is a noisy process (Suter et al., 2011), circadian rhythms in the population slowly went out of synchrony when individual clocks run slightly faster or slower (as illustrated in figure 1.5).

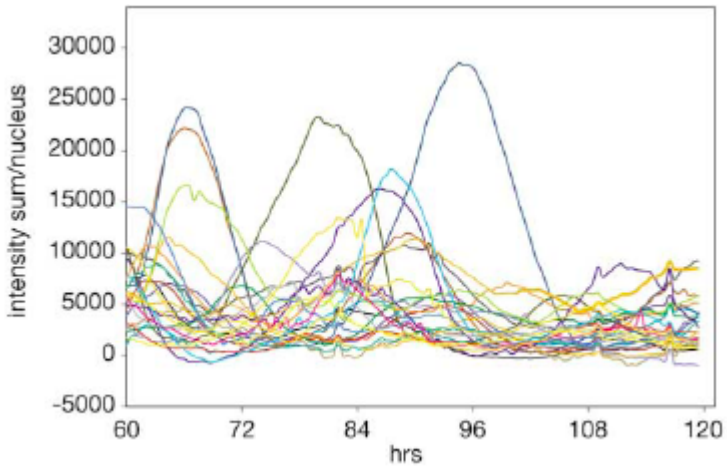

Figure 1.5 Circadian timekeeping varies between individual cells cultured in vitro (adapted from Nagoshi et al., 2004). Bioluminescence microscopy revealed that every individual cells contains a circadian clock. However, the amplitudes and period lengths are different from cell to cell. As transcription is intrinsically noisy, when the circadian oscillators are examined at individual cell level, the cell-to-cell variations become significant when compared to population studies.

The question then is how these billions of oscillators stay in synchrony inside our body? In the mammalian system, most cells are insensitive to light. About 20,000 neurons residing in the ventral hypothalamus, called the suprachiasmatic nucleus (SCN), were found to constitute the place of the master clock or pacemaker. First, stereotaxic SCN lesions render animals arrhythmic (Ibuka and Kawamura, 1975). Second, transplantation experiments of hamster SCN showed a 'temporal chimera' phenotype in locomotor activity (Vogelbaum and Menaker, 1992). Third, to elucidate whether direct neuronal connections or diffusible signals contribute to the circadian locomotor activity, encapsulated SCN neurons were transplanted into SCN lesioned hamsters. The results showed that humoral signals diffusing out from the SCN can restore the circadian locomotors activity of the lesioned animals (Silver et al., 1996). Transgenic rats with a *Per1*-luciferase transgene were the first transgenic animals used to study the synchronization of circadian clocks (Yamazaki et al., 2000). The circadian rhythmicity of SCN explants was maintained for more than 30 days while peripheral organs dampened rapidly over time. These observations suggested that the SCN neurons are "self-entraining" *in vitro*. Figure 1.6 outlined the organizations of the circadian clocks in mammalian systems.

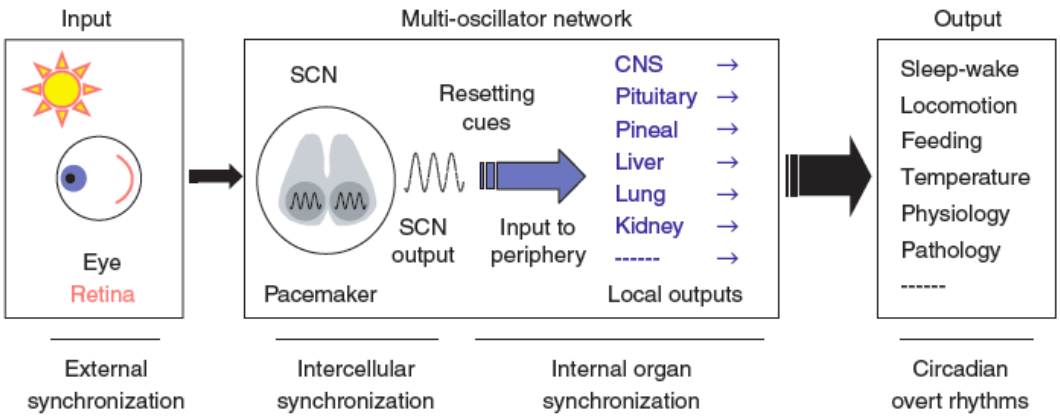

Figure 1.6 The organization of the mammalian circadian clock system (adapted from Liu, 2007). The eye (retina) is the primary sensory organs to receive external signals for the circadian clock entrainment. The photic signals from the retina are transferred to the SCN, which is the internal master pacemaker. The SCN neurons 'share time' through communications via gap junctions, neurotransmitters and electrical signals (intercellular synchronization). Neuroendocrine signals from the SCN provide the time information for the synchronization of peripheral organs like liver, lung and kidney (Internal organ synchronization). In this process the SCN signals can act indirectly by driving rest-activity and feeding fasting cycles. Virtually all physiology is circadian in mammals.

The entrainment of the circadian clock of the SCN neurons will be used as a model to illustrate different modes of signaling pathways. The SCN is entrained by daily light input from the eyes. However, the SCN does not respond to light in the same way throughout the day. No phase shift was observed when the light pulse was given during the subjective light phase. A light pulse given during the first half of the subjective night causes phase delays, while a light pulse given during the second half of the subjective night in engenders phase advances. This phenomenon is called gating (Daan and Pittendrigh, 1976)(as shown in figure 1.7).

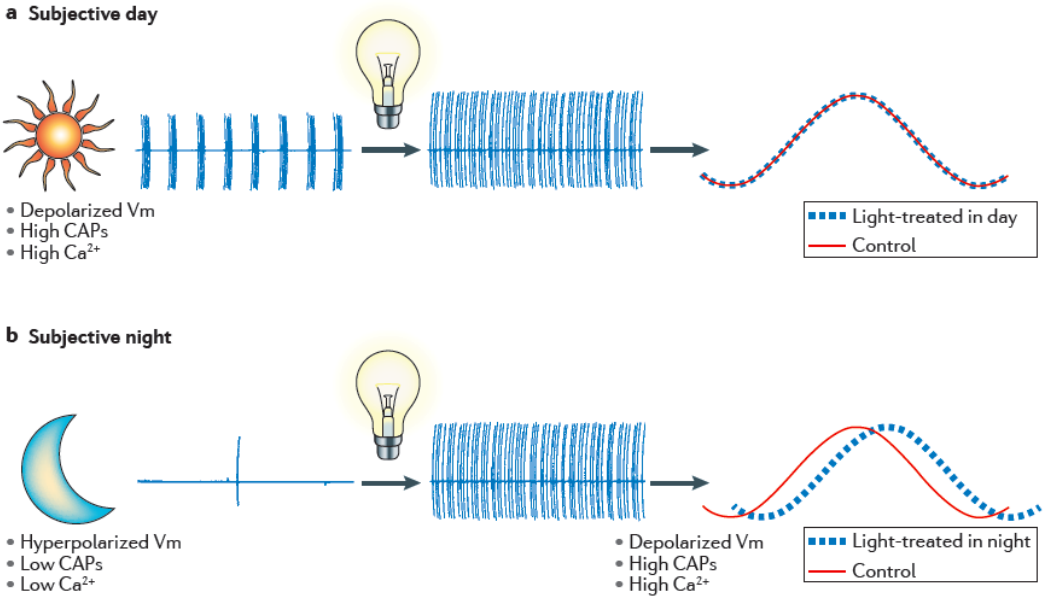

Figure 1.7 Gating of the circadian clock (adapted from Colwell, 2011). Light pulses delivered during the subjective day do not change the phase of the circadian clock of the SCN neurons. In contrast, light pulses delivered during the subjective night elicit phase delays during the first half of the night and phase advances during the second half of the night.

Melanopsin-containing retinal ganglion cells, and classical rod and cone photoreceptor cells convert the light signal into action potentials and transfer the environmental information to the SCN via the retinohypothalamic tract (Berson et al., 2002). The external signals are converted and presented internally to the cells by signal transduction pathways. Signal transduction can be considered as a three-steps process: perception of the external stimulus (signal), transfer of the signal inside the cell, and a cellular response to the stimulus. The well-characterized signaling pathways involved in photic entrainment of SCN included G-protein coupled receptors, MAP kinase, cAMP, cGMP, Ca^{2+} ion, nitric oxide (NO) and CREB. Also, there are nuclear receptors that bind to the ligand directly and activate gene expression without other proteins or mediators to transfer the signal inside the cells. The role of nuclear receptors in regulation of circadian clock will be discussed in a later part of the study.

G- protein coupled receptors (GPCRs)

G- protein coupled receptors (GPCRs) constitute one of the largest receptor family in the mammalian genome. GPCRs bind to various extracellular ligands and

transduce signals in different systems, such as the nervous system, the endocrine system, and the immune system, etc. (reviewed by Wettschureck and Offermanns, 2005). GPCRs function together with heterotrimeric G protein complexes that consist of alpha, beta and gamma subunits. The alpha subunit of the G-protein contains GTP hydrolysis activity. In the resting state, the alpha subunit is bound to GDP and associated with the beta-gamma complex. When the GPCR is activated, it promotes the exchange of GDP to GTP in the alpha subunit. The active GTP-bound alpha subunit and the beta-gamma complex dissociate from the GPCR and activate other effector enzymes or ion channels. The intrinsic GTP hydrolysis activity of alpha subunit converts GTP into GDP, which turns off the signaling event. It is common for G-proteins to activate enzymes that generate secondary messengers, for example, adenylate cyclase (AC) to generate cAMP, which in turn activates protein kinase A (PKA); phospholipase C β (PLCβ) to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2), which increases intracellular Ca^{2+} concentration and activates protein kinase C (PKC). Rho-guanine nucleotide exchange factor (Rho-GEF) regulates actin and cytoskeleton signaling by activating RhoA. There are four families of the G-protein alpha subunit, including $G_{\alpha s}$, $G_{\alpha i/G\alpha o}$, $G_{\alpha q}/G_{\alpha 11}$, and $G_{\alpha 12}/G_{\alpha 13}$ (review Wettschureck and Offermanns, 2005). The $G_{\alpha i/G\alpha o}$ family is known to inhibit the production of cAMP while the $G_{\alpha s}$ family has the opposite effect. Members of the $G_{\alpha q}/G_{\alpha 11}$ family activate PLCγ, while $G_{\alpha 12}/G_{\alpha 13}$ activates RhoA GTPase. The GPCR signaling is illustrated in figure 1.8.

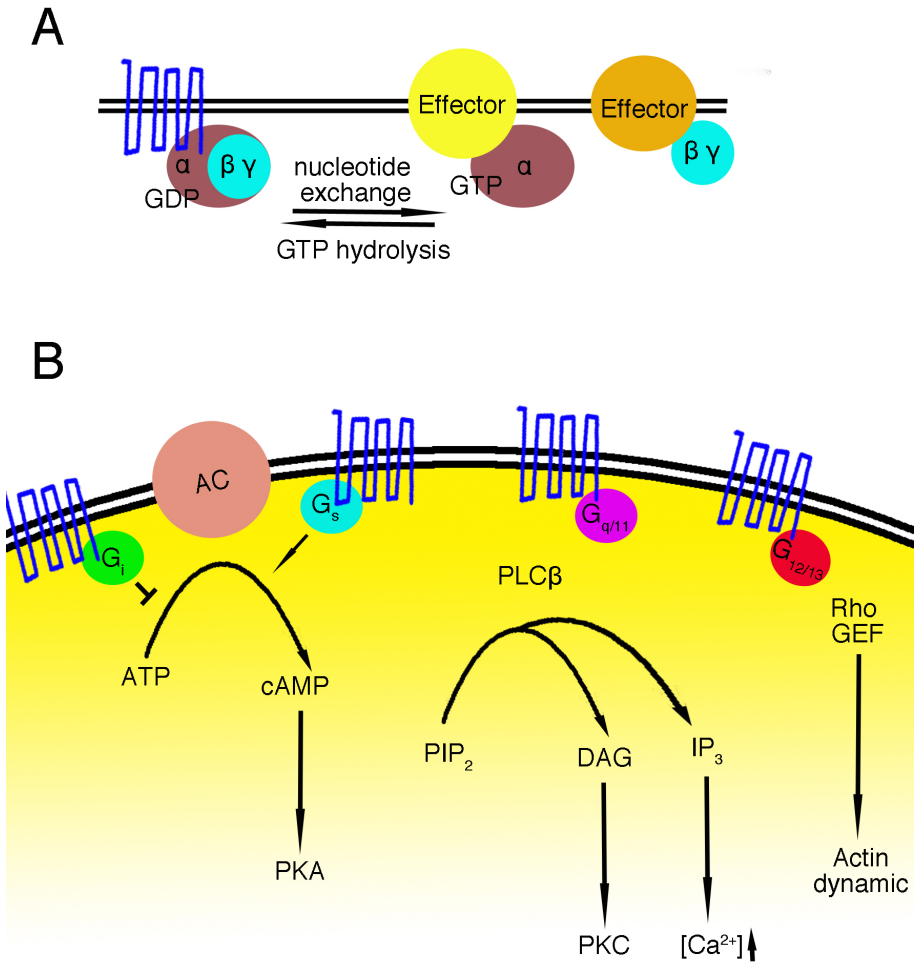

Figure 1.8 Mechanism of G-protein coupled receptor signalling pathway. A) In the resting state, G- protein coupled receptors (GPCRs) is associated with the heterotrimeric G protein complexes, GDP bound-alpha subunit and the beta-gamma subunit. When the GPCR is activated, the GDP is exchanged to GTP in the alpha subunit. The active GTP-bound alpha subunit and the beta-gamma complex dissociate from the GPCR and activate different effectors enzymes. Since alpha subunit of the G-protein contains GTP hydrolysis activity, it converts GTP back into GDP and the whole system returns back to the resting state. B) There are four main classes of the G-protein alpha subunit, including $G_{\alpha s}$, $G_{\alpha i}/G_{\alpha o}$, $G_{\alpha q}/G_{\alpha 11}$, and $G_{\alpha 12}/G_{\alpha 13}$ which caused different downstream signalling events. $G_{\alpha s}$ promote the adenylate cyclase (AC) activities to generate cAMP from ATP which activates protein kinase A (PKA). On the contrary, $G_{\alpha i}/G_{\alpha o}$ has an inhibitory effect on AC. $G_{\alpha q}/G_{\alpha 11}$ stimulates phospholipase C β (PLCβ) to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2) which increases intracellular Ca^{2+} concentration and activates protein kinase C (PKC). $G_{\alpha 12}/G_{\alpha 13}$ interacts with Rho-guanine nucleotide exchange factor (Rho-GEF) regulates actin and cytoskeleton signalling by activating RhoA.

In the nerve endings of the retinohypothalamic tract, a number of neuropeptides bind to GPCRs on the postsynaptic SCN neurons, such as vasoactive intestinal polypeptide (VIP) (Aton et al., 2005), pituitary adenylate cyclase-activating peptide (PACAP) (Vaudry et al., 2001) and gastrin-releasing peptide (GRP) (Karatsoreos et al., 2006). The mutant mice lacking VIP and VIP receptor, $VIP^{-/-}$ and $Vipr2^{-/-,}$ showed nearly arrhythmic locomotors activities under constant darkness condition, suggesting a role of this signaling pathway in keeping the SCN neurons in synchrony (Aton et al., 2005). PACAP and GRP participate in the photic entrainment of the SCN neurons, mice with mutations in these genes showed altered photic response (Kawaguchi et al., 2003; Aida et al., 2002). Dexras 1, a Ras small GTPase family G-protein, modulates the downstream signaling events after PACAP activation (Cheng et al., 2004, 2006).

Secondary messenger: cAMP

cAMP is generated by adenylate cyclase(AC) from ATP. As discussed above, AC is usually coupled with membrane receptors, such as GPCR, and activated or suppressed according to the ligand. An increase in the intracellular concentration of cAMP activates protein kinase A or cyclic nucleotide-gated ion channels. PKA phosphorylates different target proteins to bring about its effects in the cells. *In vitro*, rhythmic changes in the cAMP concentrations were observed in SCN explants *in vitro* (Prosser and Gillette, 1991). Incubation with cAMP and cAMP analogs can reset the clock in the SCN (Prosser and Gillette, 1989). *In vivo*, after activation by signals from the presynaptic end such as glutamate and PACAP in the SCN neurons, activated PKA phosphorylates transcription factors including Ca^{2+}/cAMP-reponse element binding protein (CREB) (Ginty et al., 1993). Phosphorylated CREB recruits co-activators and activates *Per1* and *Per2* transcription via binding to the cAMP response elements (CREs) in the respective promoters sequences (Tischkau et al., 2003). The higher accumulation of PER1 and PER2 then results in a phase shift by repressing the transcription of their own genes. Figure 1.9 illustrated the cAMP signaling pathway in the SCN.

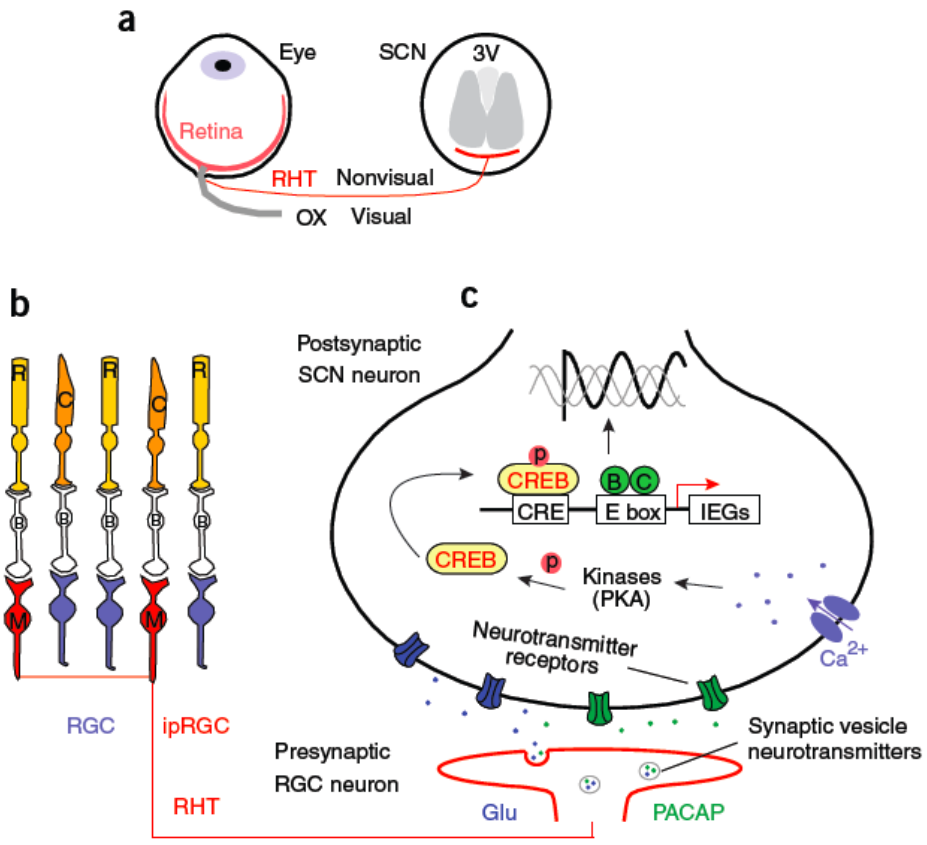

Figure 1.9 Signal transduction pathways in the photic phase entrainmment of the SCN (adapted from Liu, 2007). The environmental light/dark changes are perceived by classical rode (R) and cone photoreceptors (C) and intrinsically photosensitive retinal ganglion cells (ipRGCs) of the retina. The signals are transmitted to SCN neurons via the retinohypothalamic tract (RHT). The presynaptic RGC neurons release neurotransmitters, such as glutamate and PACAP, which trigger the influx of Ca^{2+} in postsynaptic SCN neurons. This results in an increase in cAMP and in the activation of protein kinases, including protein kinase A (PKA). The protein kinases phosphorylate transcription factors, including Ca^{2+}/cAMP-reponse element binding protein (CREB), and phosphorylated CREB binds to cAMP response elements (CREs) in the promoters of *Per1* and *Per2*, thereby activating their transcription. The sudden overexpression of PER1 and PER2 engenders a phase shift by facilitating the repression of their own genes.

Ca^{2+} ions

Ca^{2+} ions are stored outside the cell and inside the ER, so that the cytosolic concentration of Ca^{2+} is relative low in the resting state. The increase in the cytosolic concentration of Ca^{2+} can be a result of opening of ion channels or voltage-gated Ca^{2+} channels on the plasma membrane (summarized in figure 1.10). Ca^{2+} -binding proteins such as calmodulin bind to Ca^{2+} and activate other proteins like calmodulin-dependent kinase. The calmodulin-dependent kinase is likely to phosphorylate other

target proteins (reviewed by Clapham, 2007). By using calcium-binding fluorescent proteins as a reporter, calcium oscillations in SCN explants showed circadian patterns, which is important for the synchrony of the SCN neurons (Hong et al., 2012). *In vivo*, calcium signaling is important for the photic entrainment of the SCN. The retinal projections to the SCN release glutamate, which induces Ca^{2+} influx in the SCN through the opening of ryanodine receptors on the ER membrane (Ding et al., 1998). The increase of Ca^{2+} ion concentration causes phosphorylation of calcium/calmodulin-dependent Kinase II (CaMKII) (Agostino et al., 2004). Blocking calcium signaling with a calcium chelator, BAPTA-AM, or voltage-dependent calcium channel inhibitors abolished Per1 (Lundkvist et al., 2005) and *Per2* oscillations in the SCN (Nahm et al., 2005).

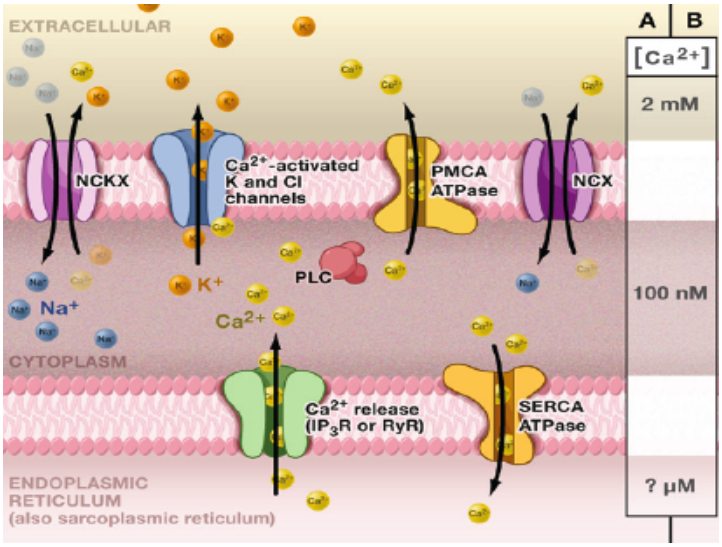

Figure 1.10 Regulation of Ca^{2+} concentration inside the cells (adapted from Clapham, 2007). There are numerous channels in the plasma membrane and in membranes of the endoplasmic reticulum, which regulate the cytosolic Ca^{2+} concentrations (NCKX: $Na^+/Ca^{2+}/K^+$ exchanger; NCX: Na^+/Ca^{2+} exchanger; PMCA ATPase: Plasma membrane Ca^{2+} ATPase; SERCA ATPase: smooth endoplasmic reticular Ca^{2+} ATPase). In the resting state, the Ca^{2+} concentration in the extracellular matrix is about 2mM while the cytosolic concentration is about 100nM. Upon the activation of G-protein coupled receptors or tyrosine kinase receptors, the intracellular Ca^{2+} concentration increases and activates Ca^{2+} ion binding proteins such as calmodulin-dependent kinase.

Nitric oxide

Beside cAMP and Ca^{2+}, nitric oxide (NO) is another important signaling molecule playing a role in the photic entrainment process. NO is generated by nitric oxide synthase (NOS) catalyzing the conversion of L-arginine (Arg) into L-citrulline

(Cit) (Ding et al., 1994). As discussed before, glutamate triggers the influx of Ca^{2+} and activates CaMKII. The NOS is phosphorylated by CaMKII (Agostino et al., 2004). NO can activate guanylate cyclase (GC), which generates cGMP from GMP. Similar to the role of cAMP in stimulating the activity of protein kinase A, cGMP activates cGMP-dependent protein kinase (cGK) activity. It is believed that CREB could be one of the downstream targets of the NO/GC pathway (Golombek et al., 2004).

MAPK pathways

In addition to GPCRs, receptor tyrosine kinases (RTK) are another significant class of cell surface receptors. RTK contain extracellular domains which dimerize when bound to their ligands. The intracellular domain contains tyrosine kinase activity, which is activated by dimerization. There are at least 20 classes of RTK transducing different signals, including as EGF, insulin, and FGF. Downstream of RTK is the MAP kinase cascade, serving in the amplification of the signal. Mitogen-activated protein kinases (MAPKs) are one of the best-studied signal transduction cascades. The basic logic of the MAPK signaling pathway goes through sequential phosphorylation events of three kinases, namely MAPK, MAPK kinase (MAPKK, MKK or MEK) and MAPKK kinase (MAPKKK) or MEK kinase (MEKK). In the mammalian system, there are at least three different groups of MAPKs, including extracellular signal-related kinase (ERK)-1/2, Jun amino-terminal kinase (JNK1/2/3), and p38 proteins (p38α/β/ρ/δ) (reviewed by Chang and Karin, 2001). As summarized in figure 1.11, different MAPKs transduce different kinds of external signals (reviewed by Goldsmith and Bell-Pedersen, 2013). Also, there are scaffold proteins like JIP1 or MP1, which bring the kinases in the proximity to their targets. This proximity provides the specificity of the signaling events (Whitmarsh et al., 2001). One of the common outputs of the MAPK signaling pathway is the phosphorylation of transcription factors. For example, MAPK phosphorylates Ets transcription factors, which induce the expression of *Fos*. FOS then dimerizes with JUN to form the activation protein 1 (AP-1) complex (Treisman, 1996). In the SCN, constitutive ERK signaling did not affect normal circadian rhythms, but it affected light entrainment (Hainich et al., 2006). During light-induced phase shifting, ERK signaling is required for the expression of immediate early response genes like *Egr-1*, *c-Fos*, and members of the Jun family (Earnest et al., 1990; Kornhauser et al., 1996). Moreover, the action

of CREB is abolished if the MAPK pathway is inhibited, while the Ca^{2+} influx is unaffected (Dziema et al., 2003). A further study suggested that Ca^{2+} acts upstream of the MAPK signaling pathway (Butcher et al., 2003).

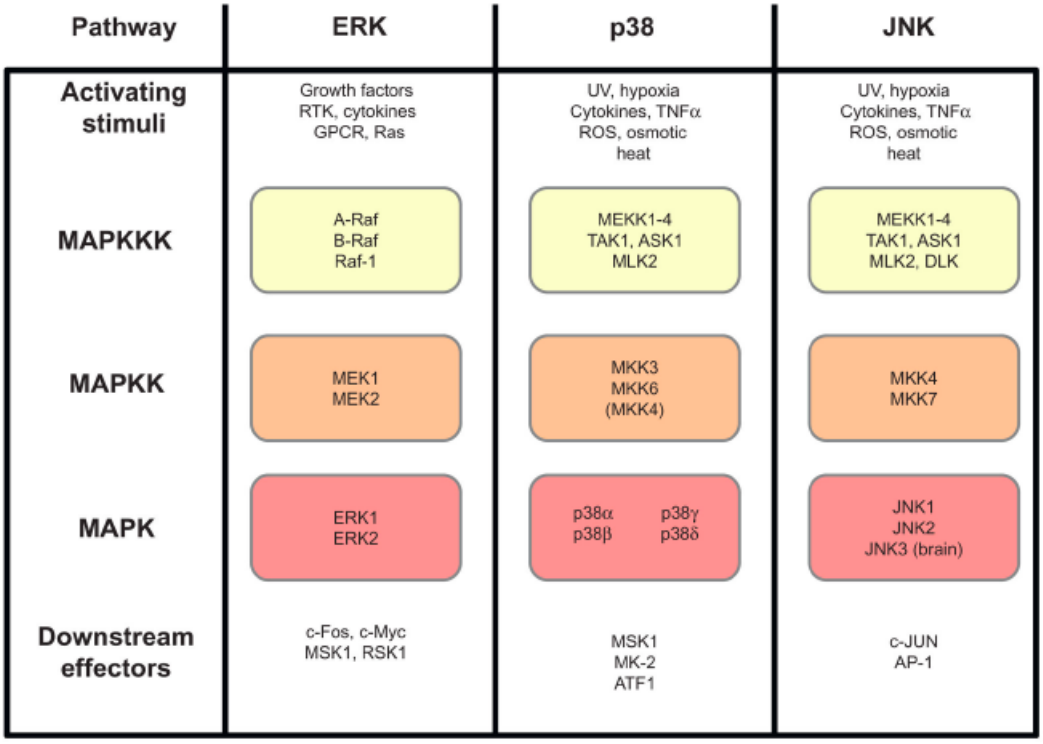

Figure 1.11 The MAPK pathways in mammals (adapted from Goldsmith and Bell-Pedersen1, 2013). The components of each pathways are outlined.

1.4 Systemic synchronization of peripheral clocks

Circadian rhythms are found in the nervous system and in virtually all peripheral organs, including liver, pancreas, heart, lung (Dunlap, 1999). Interestingly, the molecular architecture of circadian oscillators is similar if not identical in SCN neurons and peripheral tissues. There are several pieces of evidence that systemic cues are important in the phase-adjustment of peripheral clocks. First, a parabiosis study connecting SCN-lesioned mice together with intact mice showed that the liver and kidney clocks are synchronized by blood-borne factors from the wild-type partner (Guo et al., 2004). *In vitro*, circadian rhythms can be synchronized by the addition of serum to the immortalized cell lines (Balsalobre et al., 1998). A more recent study in humans found that serum from older individuals can change the clock properties of younger individual's fibroblasts (Pagani et al., 2011). Several different molecules,

such as glucocorticoid hormone or its analog dexamethasone (Balsalobre et al., 2000), retinoic acid (McNamara et al., 2001), FGF (Akashi and Nishida, 2000), TGF-beta (Kon et al., 2008) and glucose (Hirota et al., 2002) can synchronize cultured cells. Collectively, these observations suggested that many parallel or functionally redundant pathways involving blood-borne signals were implicated in the synchronization of peripheral clocks. To identify systemically regulated genes, our lab developed a transgenic mouse model in which the liver clock is 'stopped' by inducible over-expression of *Rev-Erbα* (Kornmann et al., 2007). REV-ERBα represses the expression of *Bmal1*, and thus attenuates the transcription of the genes downstream of *Bmal1*. In this system, we should be able to observe transcripts whose circadian expression is regulated by BMAL1 (i.e. by the local circadian clock) and genes whose cyclic expression is driven by systemic cues. Moreover, the transgenic model should also reveal the communications between organs, if there are changes in phase or amplitude of the circadian clock in other organs or tissue, because liver could be a source of secreting signaling molecules. Interestingly, *Per2* is one of the genes rhythmically expressed without a functional liver clock. A similar observation was later on made in liver-specific *Bmal1* knockout animals (Lamia et al., 2009), which represent a very similar experimental system. Since *Per2* itself is a component of the clock, it is believed PER2 directly relays the information from the system to the local clockwork. From the list of the systemically regulated genes, Heat Shock Factor 1 (HSF1) and Cold-Inducible-RNA-Binding Protein (CIRP) are identified, suggesting a role of body temperature in driving cyclic gene expression (Kornmann et al., 2007). In our lab, we also developed an unbiased approach STAR-PROM screening technology to screen for new signaling pathways responding to blood-borne factors (details of this technology will be discussed in the objectives and experimental design part). We discovered SRF as one of the immediate early transcription factors responding to diurnal blood-borne signals and diurnal changes in the cytoskeleton in the mouse liver *in vivo*. The daily changes in the actin cytoskeleton structures are believed to activate the Rho-actin- SRF (serum-response facto)- MRTF (Myocardin-related transcription factor) signaling pathway which synchronize the circadian clock in liver by activating *Per2* (Gerber et al., 2013).

Temperature as *Zeitgeber*

As early as in the 70s, studies in squirrel monkeys showed that the body temperature is under the regulation of the circadian system (Fuller et al., 1978). *In vitro*, clock gene expression can be entrained by imposing simulated body temperature cycles (Brown et al., 2002; Saini et al., 2012). *In vivo*, the higher environmental temperature can also cause phase shifts in the liver, but not in the SCN (Brown et al., 2002). Subsequently, research in Takahashi's group revealed that the connections between SCN neurons are necessary to resist the effect of temperature. When they treated the SCN explants with drugs like a voltage-gated Na+ channel blocker, tetrodotoxin and a L-type calcium channel blocker, nimodipine, the circadian rhythms in SCN can suddenly respond to temperature (Buhr et al., 2010). Heat Shock Factor 1 (HSF1) is found to be a transcription factor that participates in relaying temperature information to the circadian clock (Buhr et al., 2010; Saini et al., 2012). In addition, cold-inducible RNA-binding protein (CIRP) plays a role in temperature entrainment by modulating the amplitude of the molecular oscillator. At least in part this is accomplished by controlling the expression of CLOCK (Morf et al., 2012).

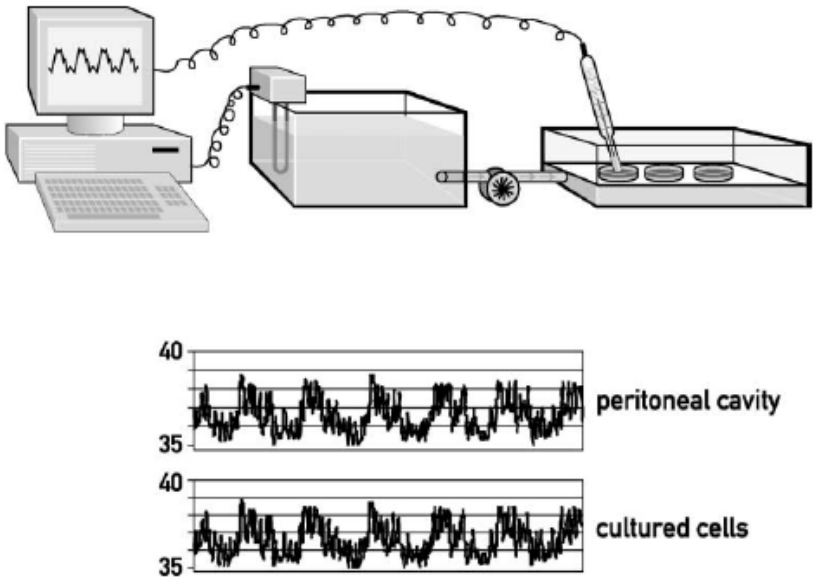

Figure 1.12 Simulated body temperature rhythms can entrain cells *in vitro* (adapted from Brown, 2002). In our lab, we have developed a computer-assisted incubator capable of imposing body temperature rhythms on cultured cells. Such simulated body temperature (recorded in mice) can efficiently synchronize circadian gene expressions in cultured cells.

Food as *Zeitgeber*

At the cellular level, it appears that there are numerous connections between metabolism and circadian clocks (Asher and Schibler, 2011). For instance, BMAL1 was found to be O-GlcNAcylated (Durgan et al., 2011), which affected its transcription activity. Since O-GlcNAcylation of proteins is often associated with the availability of glucose (Kamemura and Hart, 2003), it is possible that O-GlcNAcylation of BMAL1 is under the control of glucose levels inside the cells. PARP-1, a NAD^+-dependent ADP-ribosyltransferase, poly(ADP-ribosyl)ates CLOCK under the regulation of food intake (Asher et al., 2010). Co-factors like NADH, generated by glycolysis, the TCA cycle can reflect the energy status of the cells. The pool of NAD^+/NADH also depends on the de novo synthesis of NAD^+. NAD^+ and its biosynthetic pathways, including the rate-limiting enzymes nicotinamide phosphoribosyltransferase (NAMPT), display circadian rhythms in the liver and cultured fibroblasts whose oscillators were synchronized *in vitro*. NAMPT expression is indeed under the control of the circadian clock, and the inhibition of NAMPT promotes *Per2* expression. The NAD^+ -dependent deacetylase SIRT1 is required for the maximal expression of the circadian clock genes *Bmal1* and *Ror*. BMAL1/CLOCK interacts with SIRT1 and is thought to regulate the acetylation of BMAL, PER2, and histones (Asher et al., 2008; Nakahata et al., 2008; Ramsey et al., 2009; Nakahata et al., 2009).

At the systemic level, the possible role of food as a *Zeitgeber* in mammals was first suggested in the 70s, for example, circadian changes in liver phosphorylase activity (Vilchez et al., 1974) and corticosteroid levels in the blood are altered in food restricted conditions (Krieger, 1978). Furthermore, changes in circadian wheel-running activity (Edmonds, 1977) were observed when food access was restricted. Our group pioneered a study on the effect of feeding cycles on the peripheral clocks (Damiola et al., 2000). They showed that in the SCN the phase remained locked to the light-dark cycle, however, in liver and other peripheral tissues, the phase of the circadian gene expression depended on the feeding schedule (Damiola et al., 2000). Similar conclusions were drawn from a study in Menaker's laboratory (Stokkan et al., 2001). Several feeding-related endocrine molecules showed diurnal rhythms in the blood that may have implications for the regulation of circadian clocks in peripheral

organs. These include adiponectin, insulin, leptin, prolactin, ghrelin and FGF21 (reviewed by Gamble et al., 2014).

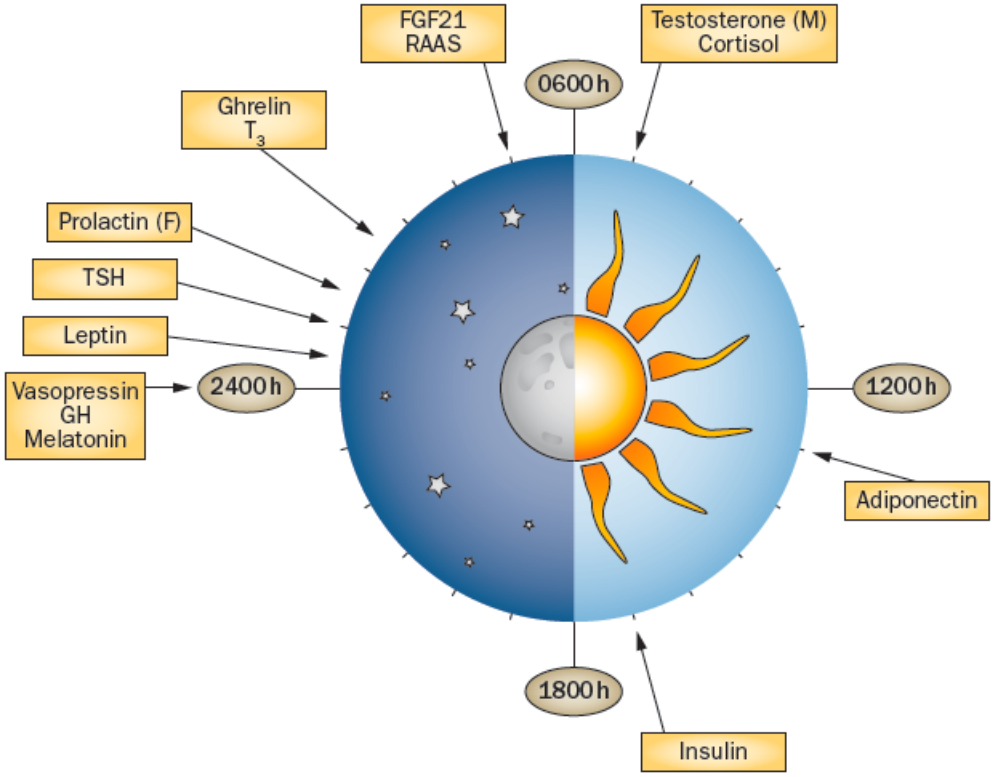

Figure 1.13 Diurnal endocrine factors in humans (adapted from Gamble, 2014). F: Female only; M: male only; FGF21: Fibroblast growth factor 21; GH: growth hormone; RAAS: renin-angiotensin-aldosterone system; TSH: Thyroid-Stimulating hormone.

Blood-borne factors as *Zeitgebers*

Glucocorticoid secretion shows a robust diurnal rhythm in the blood that is under the control of the hypothalamic-pituitary-adrenal (HPA) axis. The corticotropin-releasing hormone regulates the release of the adrenocortiotropic hormone (ACTH), which controls the release of cortisol in the adrenal gland. In addition to the control of HPA, the 'local' circadian clock in the adrenal gland also regulates the release of glucocorticoids (reviewed by Gamble et al., 2014). Glucocorticoids can phase shift circadian gene expression in the liver (when injected intraperitoneally into mice) or in cultured cells (Balsalobre et al., 2000). However, it does not act as a 'feeding-related' signal, as it actually inhibits food-induced phase shifting of peripheral clocks (Le Minh et al., 2001).

Ghrelin, a 28 amino acid peptide secreted from the stomach, was first identified as an endogenous ligand of the G-protein coupled receptor, GHS-R, which stimulates the release of growth hormone from the pituitary (Kojima et al., 1999). Ghrelin is an important hormone in regulating appetite. This obviously implicates a role of Ghrelin in obesity and other diseases related to feeding (Nakazato et al., 2001). Studies suggested that there is a role of ghrelin in the regulation of the circadian system. First, ghrelin can cause phase shifts in the circadian gene expression of SCN explants. However, *in vivo* it only displayed effects when injected into the brain of food-deprived mice (Yannielli et al., 2007). Second, under constant light conditions (DD), GHSR-knockout mice showed an increase in overall locomotor activity, a lengthening of the period length, and a higher resilience to the rhythm degenerating effect of DD (Lamont et al., 2014). Third, circadian rhythms of ghrelin-containing cells in the stomach, the oxyntic cells, are entrained by food-related signals instead of light, suggesting a regulation mechanism of ghrelin secretion in response to food (LeSauter et al., 2009). Another hormone, oxyntomodulin, secreted from the gut, was also shown to have a resetting ability of the liver circadian clock-mediated by feeding (Landgraf et al., 2015).

The role of other metabolism-related hormones in regulation of the circadian clock have yet to be elucidated. One of the difficulties of studying the effect of food is the large number of animals that have to be sacrificed for such kinetic studies. Obviously, this renders the screening for new signal transduction components difficult. In our lab, Saini et al. developed an *in vivo* bioluminescence monitoring system, dubbed RT-Biolumicorder, which can measure gene expressions, locomotors activities, and drinking rhythms in real time. These studies have demonstrated that in SCN-leasoned mice the phase of the circadian clocks in the liver adapted faster to restricted feeding regimens than that of wild-type animals (Saini et al., 2013).

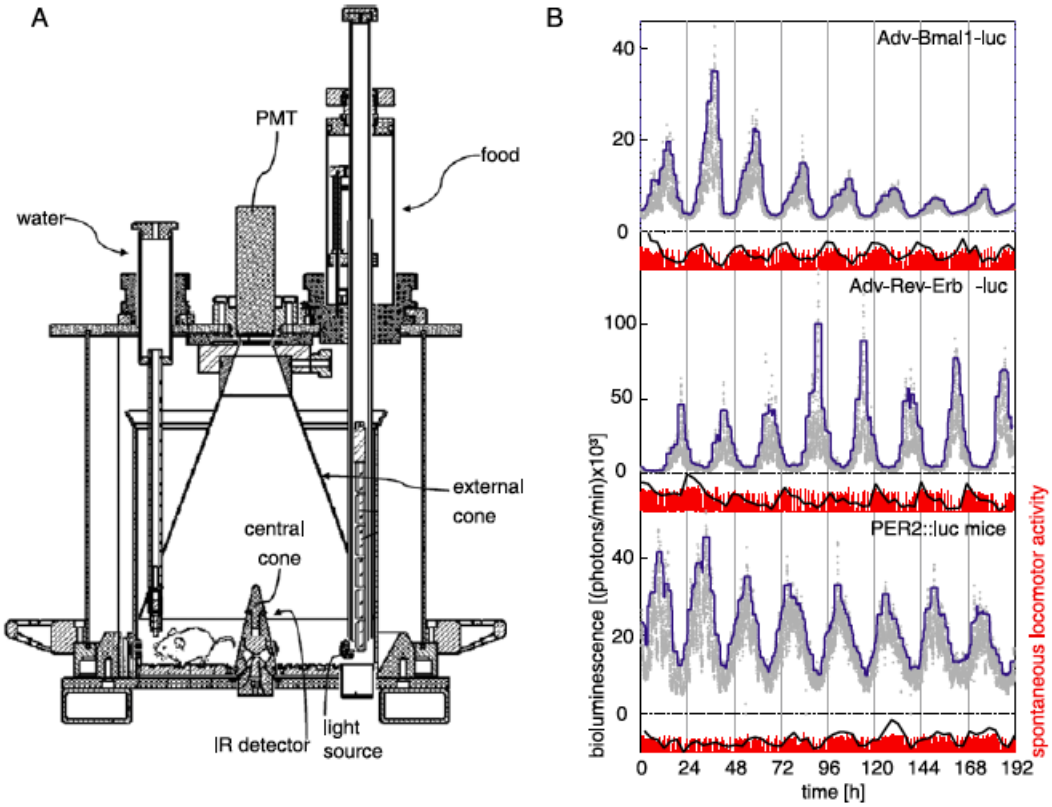

Figure 1.14 The RT-Biolumicorder, an *in vivo* bioluminescence measuring system (adapted from Saini et al., 2013). The system can be used to monitor bioluminescence of mice expressing luciferase reporter genes (for example mice whose liver cells were transduced with adenoviral vectors harbouring circadian luciferase reporters). Skeleton photoperiods (for the light entrainment of mice) and food availability can be computer-controlled according to the experimental design. The locomotors activity cycles (which are a reliable readout for the activity of the SCN) can also be recorded in real time by an infrared (IR) detector.

1.5 Objectives of thesis

Identification of immediate early transcription factors and signaling pathways possibly involved in synchronization of circadian clocks in peripheral tissues.

1.6 Rationale and experimental design

The expression of immediate early transcription factors (IETFs) like c-FOS, PER1 and PER2, is rapidly induced in cultured cells after the serum shock (REF). Based on this observation we hypothesized that clock synchronization is achieved via direct or indirect interactions among IETFs and core clock components (as shown in figure 1.15). The synthetic tandem repeat promoter (STAR-PROM) screening, originally developed by Gerber et al. in our laboratory (Gerber et al., 2013), was performed to identify individual IETFs in the osteosarcoma U2-OS cells. The logics of the STAR-PROM technology is based on a method previously designed in our laboratory, dubbed Differential display of DNA-binding protein method (DDDP) (Reinke et al., 2008). According to the statistics, we could estimates the size of a collection of random DNA sequences to contain the binding sites of most of the transcription factors. The details of the statistics are shown in figure 1.16. By designing different screening strategy, we can fish out different transcription factors. For example, DDDP was designed to look for transcription factors showing circadian protein-DNA binding pattern *in vitro*.

STAR-PROM directly assays for transcriptional activity in response to blood-borne factors rather then for DNA-binding affinity. The bioluminescence produced a by short-lived nuclear luciferase under the control of STAR-PROM promoters is measured as the readout of the transcription induction (Suter et al., 2011). At least five repeats of a transcription factor binding sites (i.e. cAMP-responsive elements) are required to yield strong reporter gene induction (Gerber et al., 2013). Synthetic tandem repeat promoters are generated by rolling circle amplification of random-mers. The multi-mers were cloned into a luciferase reporter plasmid upstream of a minimal promoter encompassing a TATA-box. I adapted the procedures to a lentiviral vector delivery system, because lentiviral vectors are well-established tools for generating stable gene transfers into dividing and non-dividing cells. The lentiviral library would allow me to screen for IETFs induced by blood-borne factors in

different cell types or even tissue pieces. In this study, HepaRG cells were used for the screen.

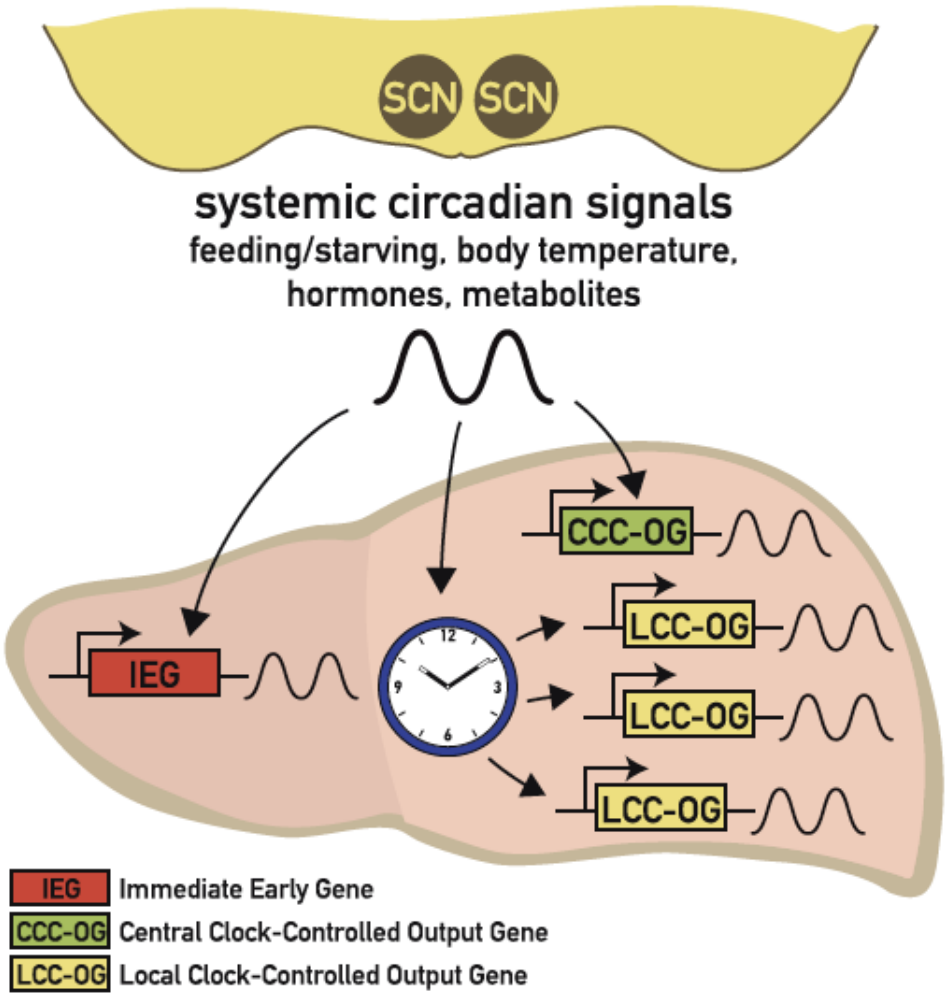

Figure 1.15 Our working model for the systemic control of circadian gene expression in peripheral organs (adapted from Asher and Schibler, 2011). Systemic circadian signals controlled by the SCN drive the peripheral clocks by directly acting on clock-controlled gene (e.g. genes encoding heat shock proteins), immediate early genes (IEGs) (e.g. *c-Fos*), and immediate early genes that are also core clock components (e.g. *Per2*).

A

(1) $$F_{0-n} = \frac{2}{4^N} \sum_{i=0}^{n(\leq N)} \frac{N!\,3^i}{i!(N-i)!}$$

(2) $$p = 1-(1-F_{0-n})^Z$$

B

For Z = 55,000 bp

N	p		
	m = 0	m = 1	m = 2
7	0.999	>0.999	>0.999
8	0.813	>0.999	>0.999
9	0.343	>0.999	>0.999
10	0.100	0.961	>0.999
11	0.026	0.590	>0.999
12	0.007	0.215	0.984

Figure 1.16 Statistical basis of STAR-PROM (adapted from Gaber et al, 2013) A) The Pascal's binominal theorem (Equation no. 1) was used to calculate the statistical frequency (F_{0-n}) of a hypothetical transcription factor binding (N bp long), with the assumptions that the binding site is non-palindromic and containing 0 and n mismatches (i represent different values of n). The probability (p) of discovering the transcription factor binding sites at least once in a given length of random DNA (Z) can be determined by Equation no. 2. (B) According to the equation no. 2, the probabilities (p) of finding different transcription factor binding sites are presented in the table with the random DNA library size equals to 55'000 bp, with 7 to 12 bp (N) and allowing for n mismatches. There is larger than 99.9% of finding a transcription factor with a consensus sequence of 11 bp and with 2 mismatches.

HepaRG is a bipotent liver progenitor cell line (Parent et al., 2004) which can be differentiated hetpatocytes. It was derived from a liver tumor from a female patient. The HepaRG cells exhibit stem cells properties, for example, when the cells are seeded at low density, they undergo active cell divisions until they are confluent in the culture dish. This process takes about one week. The confluent culture stops cell

division and differentiates to form hepatocytes and biliary cells. The culture can be stabilized by addition of 2% DMSO in the culture medium. DMSO is toxic to the progenitors cells, so it kills off the cells that are not fully differentiated. Also, it can induce the expression of genes related to xenobiotic detoxification. The cells can be propagated by trypsinizing them and seeding them at a low density. The mature hepatocytes undergo dedifferentiation, going back to the stem cell-like state, and restart cell division (Cerec et al., 2007). Transcriptome and toxicology studies support the similarity between HepaRG cells and primary human hepatocytes (Kanebratt and Andersson, 2008; Hart et al., 2010). The HepaRG cells hence offer an alternative to human primary hepatocyte cultures, and, in addition enhance reproducibility by eliminating individual variations between different donors.

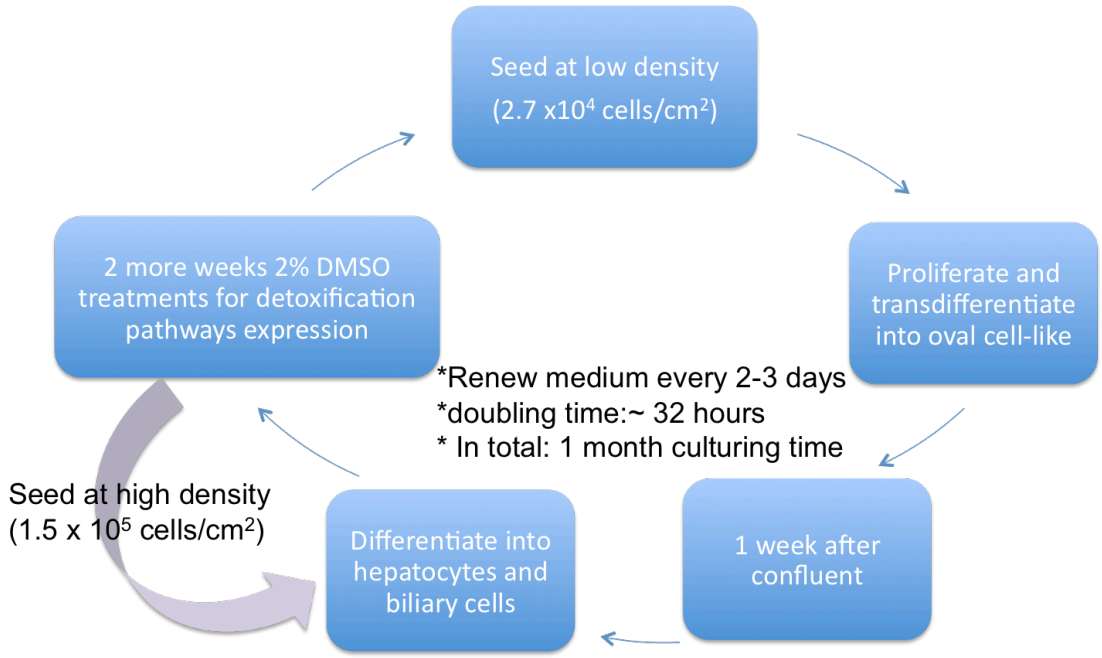

Figure 1.17 The preparation of differentiated HepaRG cells. HepaRG cells are seeded at low density. The cells proliferate as oval cell-like. Once confluent, the cells differentiate into hepatocytes and billary cells. The culture of differentiated cells can be stabilized by treatment with 2% DMSO. The differentiated cells can return back to the proliferative state when trypsinized and seeded at low density, while the cells stay differentiated when seeded at high density.

2. Results

Impact of FOXA2 on the mouse liver circadian clock (Manuscript)

Impact of FOXA2 on the mouse liver circadian clock

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ABSTRACT

Circadian clocks enable organisms to anticipate and to adjust to daily changes in the environment. To provide optimal coordination of the circadian clocks, the system has to resynchronize its individual parts continuously. Hence, systemic cues such as body temperature and yet to identify signaling molecules in the blood are necessary to coordinate circadian oscillators of peripheral organs. This synchronization is probably mediated by transcription factors responding rapidly to these cues to affect clock gene expression. Here, a modified *Synthetic tandem repeat promoter* (STAR-PROM) screen was used to identify novel serum-responsive, immediate early transcription factors in a differentiated hepatocyte cell line, HepaRG cells. Among the obtained candidates FOXA2 was found to respond to blood-borne factors. Surprisingly, the thrombin inhibitor hirudin reduced the diurnal activation of FOXA2 by serum. Therefore, besides other signaling pathways such as Ca^{2+}, MEK1/2 and tyrosine kinases, thrombin activated FOXA2 probably via the Protease-activated receptor 1 (PAR1). The siRNA-mediated knockdown of *FoxA2* in a HepaRG caused dampening of circadian clock. Furthermore liver-specific, inducible knockout mice uncovered the role of FOXA2 in the circadian clock *in vivo*. Upon inactivation of FOXA2, the expression of a *Rev-Erbα*-driven luciferase reporter in the liver was attenuated over the course of two weeks without affecting the total locomotors activity. This phenotype was confirmed by measuring clock gene expression in the liver of these mice. Finally, chromatin immunoprecipitation (ChIP) experiments performed on the *Rev-Erbα* genomic locus suggested that FOXA2 depletion might affect the local chromatin structure at the promoter. In summary, we have found the activity of FOXA2 was induced by serum signals and that FOXA2 plays an important role in sustaining circadian clock function *in vivo*.

INTRODUCTION

Circadian clocks provide a proactive mechanism for the organism to predict periodic alterations in the environment, such as the daily light and dark cycles (Albrecht, 2012). These clocks also allow species to occupy different temporal ecological niches while sharing the same environment. For instance, nocturnal animals are active during the night while diurnal animals are active during the day. Circadian clocks generate daily waves of gene expression that govern rhythmic changes in metabolism, physiology and behavior.

At the molecular level, the circadian rhythms of mammals are thought to be generated by two coupled transcriptional/translational feedback loops and post-translational modifications of the core clock components (Ko and Takahashi, 2006). In the primary feedback loop, there are the positive limb consisting of *Arntl*/*Bmal1* and *Clock*, and the negative limb composed of *Periods* (*Per1 and Per2)*) and *Cryptochromes* (*Cry1 and Cry2*). The transcription factors BMAL1 and CLOCK activate the expression of *Per1/2*, *Cry1/2* and *Rev-Erbα/β* (official names of these two orphan nuclear receptors: Nr1d1 and Nr1d2). Once protein complexes formed by the interaction of PERs and CRYs reach a critical concentration, they bind to CLOCK-BMAL1 and thereby annul its transactivation potential. This results in the repression of *Per1/2, Cry1/2,* and *Rev-Erbα/β* transcription. REV-ERBα and Rev-ERBβ, the negative limb members of secondary feedback loop, compete with members of the RORα/β/γ orphan receptor family, the positive limb members of the secondary feedback loop, for the binding to RORE elements in the promoters of *Bmal1* and *Clock*. Due to the action of REV-ERBα/β the cyclic expressions of *Bmal1* and *Clock* are nearly anti-phasic to that of *Per* and *Rev-Erb* expressions. Clock-controlled output genes, which govern overt rhythms in behavior and physiology, are linked to these core clock circuitry either directly, or indirectly, by using transcription factors like PAR domain basic leucine zipper proteins (PAR bZip), whose rhythmic accumulation is regulated by core clock components (Ripperger and Brown, 2010).

In mammals, almost all the cells in the body contain circadian clocks with similar molecular components. However, a master clock resides in the *Suprachiasmatic nuclei* (SCN) within the brain, which directly receive light information from the eyes (Moore, 1983). The peripheral organs like liver, heart, lung and kidney harbor "slave clocks", which need signals directly or indirectly from the SCN to tell the time of the day (Dibner et al., 2010). The body temperature is a typical systemic phase-setting cue in mammals (Buhr et al., 2010). It can synchronize the peripheral oscillators but due to the network characteristics of the SCN this structure is resilient to the effects of body temperature changes. Experiments done by Guo and colleagues suggested the involvement of blood-borne factors in synchronization of peripheral clocks in the liver and kidney, but not heart, spleen, and skeletal muscle (Guo et al., 2005). The surgical removal of the SCN caused desynchronization of their circadian oscillators. Synchronization was subsequently restored in liver and kidney by parabiosis with SCN-lesioned mice. This resynchronization provided strong *in vivo* evidence for the participation of blood-borne factors in the synchronization of peripheral clocks. Nevertheless, despite many years of research the nature of these synchronizing factors in the blood and the corresponding signaling pathways remain to be identified.

In vitro, the clocks of cultured cells can be transiently synchronized by a puzzling array of reagents, including serum, TGF-β (Kon et al., 2008), cAMP (e.g., forskolin), protein kinase C agonists, glucocorticoid hormones, Ca^{2+} ionophores (Balsalobre et al., 2000), and glucose (Hirota et al., 2002). This redundancy in signaling renders the task to identify specific factors challenging. Nonetheless, the phase entrainment of cellular clocks by all of these pathways involved the activation of immediate early transcription factors (IETFs). For instance, the activation of the cAMP-signaling pathway results in the phosphorylation of the CREB protein, which in turn induces *Per1* expression and, subsequently, a phase-shift of the circadian oscillator (Gau et al., 2002; Travnickova-Bendova et al., 2002). Since IETFs appeared to induce *c-Fos*, *Per1* and *Per2* in cultured cells after a serum shock (Balsalobre et al., 1998), we hypothesized that the synchronization of peripheral clocks was achieved by the activation of IETF via blood-borne signal(s). Previously a *Synthetic tandem repeat promoter* (STAR-PROM) screening technology was developed to identify serum-responsive IETFs (Gerber et al., 2013). This resulted in the discovery of Serum

response factor (SRF) and its coactivators MRTF as the mediators of circadian clock synchronization. While the original STAR-PROM screening technology is applicable to easily transfectable cell lines, it cannot be used efficiently for cell types that are difficult to transfect, such as differentiated cell lines or primary cells.

In this study, we modified the method by constructing the STAR-PROM library using lentiviral vectors and successfully performed the screen in differentiated human hepatocytes derived from HepaRG cells (Barraud et al., 2005). Serum response element (SRE) containing clones were still among the most prominent serum-induced clones. However, perhaps due to the larger size of the library and/or the different cell type used, a number of new IETF candidates were identified, including FOXA2. Here, we report on how FOXA2 is activated and on the functions this transcription factor may play in the circadian timing system.

RESULTS

Construction of the modified STAR-PROM library and design of the screen

In this study, we aimed to discover liver-specific signaling pathways affecting the circadian oscillator. The original STAR-PROM screening method (Gerber et al., 2013) was thus modified as follows. First, the library was constructed into a lentiviral vector, driving the expression of a short-lived luciferase (Suter et al, 2011). We named the new vector pSTAR (supplementary figure 1). Lentiviral vectors stably transfer genes into a whole range of dividing and non-dividing cells, and their use therefore extends the number of cell types suitable for the screen. Second, we have increased the size of the library to more than 2,800 clones, which samples nearly 400,000 bp of random DNA for the presence of transcription factor binding sites. This library is about 3-times more complex than the one used in the previous screen. (The details of the cloning procedures of the library please refer to materials and method and supplementary figure 2 and 3) Third, we have chosen HepaRG cells, a differentiated human hepatic cell line, for this study.

The HepaRG cells can be differentiated into hepatocytes resembling in many aspects human primary hepatocytes. We initially tested whether HepaRG cells displayed robust circadian rhythms by transducing them with a Bmal1-luc lentiviral

vector and selecting stable clones. After 6 days in medium with 2% DMSO to differentiate the HepaRG-Bmal1luc cells into hepatocytes (Cerec et al., 2007), a serum shock was performed and bioluminescence monitored continuously (Nagoshi et al., 2004). The reporter cell line showed robust rhythmic expression of luciferase driven by the *Bmal1* promoter for at least five cycles (figure 1A). This result supported the use of differentiated HepaRG cells as model system to study circadian gene expression in hepatocytes. To generate a positive control for the screen, 8 copies of SRF binding sites were inserted into the pSTAR lentiviral vector and stable clones selected. The differentiated HepaRG-SRF cell line showed a robust response to a serum shock (figure 1B). Consequently, differentiated HepaRG cells can also be used for screening immediate early response factors.

The primary screen intended to identify serum-responsive clones using a high-throughput screening protocol. The positive clones were individually re-tested to eliminate false positive clones in a secondary screen. In the primary screen, 2,861 clones of differentiated HepaRG cells were screened with 4% human plasma. 30 positive clones, corresponding to about 1% of the whole library, were obtained and verified for their response to human plasma. Among those clones, 8 were responsive to forskolin-, 1 to dexamethasone, 2 to glucose (100 mM), and 5 to signals inducing SRF.

Characterization of the clone p11D7 as an immediate early response clone

In the primary screen, we identified the clone p11D7 (96-well plate no. 11, D7 position) showing robust induction of luciferase activity by serum (figure 2A). We verified that this induction was due to an increase of luciferase expression as opposed to an increase in luciferase activity by treating the cells with actinomycin D and cycloheximide, which block RNA and protein synthesis, respectively. Both treatments abolished the luciferase signal. An immediate early response is a response not requiring *de novo* protein synthesis for the induction of gene expression. To verify whether clone p11D7 is mediating an immediate early response, the HepaRG-p11D7 reporter cell line was pre-treated with actinomycin D or cycloheximide before the serum shock, and RNA samples were collected at different time points afterwards (figure 2B). We found that the accumulation of luciferase mRNA did not rely on *de novo* protein synthesis. Therefore, p11D7 is a true immediate early response clone. In

the secondary screen, the HepaRG-p11D7 reporter cell line showed a diurnal induction pattern when induced by human plasma (figure 2C and 2D) rat serum (figure 2E) and collected around the clock.

The transcription factor binding sites within the synthetic promoter of clone p11D7 was identified by an electrophoretic mobility shift assay (EMSA) (figure 3A). The synthetic promoter element was 68 nucleotides long, which for the EMSA experiments was divided into three parts, each 23 nucleotides long. Nuclear extracts were prepared from HepaRG cells at 0, 1, 2 and 3 hours after serum shock and incubated with the (^{32}P)-labeled oligonucleotides. Protein:DNA complexes were observed, the intensity of which increased slightly 1, 2 and 3 hours after the serum shock. To ensure that probe 3 contained the serum-responsive site, we constructed three new HepaRG cell lines for each of the sites for functional tests. Consistent with the EMSA results, the clone containing the sequence of probe 3 showed a robust serum response while the clones containing the sequences of probe 1 and probe 2 were not serum responsive (figure 3B). Hence, a cis-acting element responsive to a blood-borne cue was located in probe 3.

FOXA2 is the transcription factor mediating the serum-response of p11D7

There is a putative HNF3B/FOXA2 binding consensus sequence found in probe 3, as identified using the motif search program Patch 1.0. Consequently, we used siRNA against *FoxA2* to examine whether FOXA2 is indeed responsible for the serum induction of clone p11D7. As shown in figure 4A, the induction of p11D7 expression in differentiated HepaRG-p11D7 cells was decreased when *FoxA2* was knocked down. Since FOXA1 and FOXA3 were expressed in the HepaRG cell line, and they can bind to similar sequences, we also used siRNAs to knockdown *FoxA1* and *FoxA3*. There were no significant decreases in the serum response. A RT-qPCR analysis showed that the siRNA reduced quite specifically the expression of *FoxA1*, *FoxA2* and *FoxA3* despite the fact that these three transcription factors share sequence homology (figure 4B). Nevertheless, there was some down regulation of *FoxA1* when *FoxA2* was knocked down. This result may indicate some cross-reactivity of the siRNA, or cross-regulation between FOXA1 and FOXA2. The knockdown of *FoxA2* by siRNA greatly reduced the diurnal response of HepaRG-p11D7 to serum all over

the day (figure 4C). As shown in figure 4D, treating the HepaRG cells with siRNA successfully diminished FOXA2 accumulation in nuclear extracts. These experiments characterized FOXA2 as a novel serum-responsive immediate early transcription factor.

Characterization of signaling pathways affecting clone p11D7

In order to find out which signaling pathway(s) were responsible for the serum response mediated by FOXA2, different drugs were used to block known signaling pathways. First, we tested if individual drugs can affect FOXA2 activity in the HepaRG-p11D7 reporter cell line. As shown in figure 5A, the HepaRG-p11D7 reporter cell line can be induced by increasing the intracellular Ca^{2+} concentration by treatment with ionomycin. On the other hand, the inhibition of signaling by MEK1/2 kinase (U0126), protease-activated receptor 1 (PAR1) (SCH79797), actin polymerization (latrunculin B and cytochalasin D), PI_3 kinase (wortmannin), and tyrosine kinase (genistein) signaling did not induce HepaRG-p11D7 reporter expression. We then pre-treated the HepaRG-p11D7 reporter cell line with the drugs for 1 hour before the serum shock. We expected the induction would be reduced when blocking the signaling pathway that participates in the serum response mediated by FOXA2. The control was cells pretreated with the solvent DMSO for 1 hour, before the serum shock. Usually, about 20-fold induction was obtained with 1% rat serum. The fold-inductions of all the different drug treatments were normalized to control (DMSO). As shown in figure 5B, inhibition of MEK1/2 kinase, PAR1 and tyrosine kinase reduced the induction by serum. However, these observations also suggested that there were more than one potential inducers activating FOXA2. To further characterize potential inducing factors in the serum, trypsin was used to cleave most of the proteins present (figure 5C). Treatment of the serum with this protease reduced the serum response of HepaRG-p11D7, which was partially prevented in the presence of a specific trypsin inhibitor (figure 5D).

Thrombin as a novel inducer of FOXA2

Since thrombin can activate PAR1 and since this blood-coagulating component is known to have signaling activity (Coughlin, 2000), we tested whether it can induce the HepaR-p11D7 reporter cell line. A dose response experiment was performed with increasing amounts of thrombin to induce the HepaRG-p11D7 reporter cell line

(figure 6A). As low as 0.5 NIH units of thrombin was sufficient to induce luciferase expression. The addition of the specific thrombin inhibitor hirudin reduced the induction of HepaRG-p11D7 cells by rat sera collected around the clock (figure 6B). However, the diurnal pattern was still observed. In a crude protein separation process of rat serum by ammonium sulfate precipitation, thrombin was enriched in fractions obtained by 50% and 60% ammonium sulfate saturation. However, all other examined fractions could activate the reporter to some extent (figure 6C). These results suggested there are other proteins in the serum that can induce FOXA2. Since the cAMP pathway is known to regulate FOXA2, we tested if thrombin interacted with this pathway (figure 6D) (Cheng et al., 2006). When the cells were pretreated with the adenylyl cyclase stimulant forskolin, which increases the concentration of cAMP, the induction by thrombin decreased with increasing concentrations of forskolin. Blocking cAMP production by a non-competitive adenylyl cyclase inhibitor, 9-Cyclopentyladenine monomethanesulfonate (9-CP-Ade mesylate), moderately increased the induction of luciferase expression by thrombin. Taken together, these observations suggested that there is an antagonistic relationship between cAMP and thrombin signaling on the activation of FOXA2.

Interestingly, the effect of thrombin on HepaRG cells was not restricted to the activation of FOXA2. We treated two different cell lines harboring circadian reporter genes, HepaRG-Bmal1luc and HepaRG-Per2luc with thrombin. In the control, the circadian expression of both reporter cell lines dampened over time, while the addition of thrombin after day 3 resynchronized luciferase expression in both HepaRG-Bmal1luc and HepaRG-Per2luc cells (figure 6E and 6F). Furthermore, thrombin could also induce other positive clones found in the screen, including the positive control HepaRG-SRF (Supplementary figure 4). This observation suggested that thrombin had a rather broad signaling effect on the cells.

Conditional knockout of *FoxA2* caused attenuation of the circadian clock *in vivo*

In vitro, the knockdown of *FoxA2* caused the rapid dampening of the circadian clock reporter genes in the stably transduced HepaRG cell lines, HepaRG-Bmal1luc and HepaRG-Per2luc (figure 7A and 7B). To study the role of FOXA2 for the regulation of the circadian clock *in vivo*, we have performed *in vivo* imaging experiments on hairless mice carrying an albumin promoter-driven, tamoxifen-

inducible Cre-recombinase gene and floxed *FoxA2* alleles. In these mice, the hepatocyte-specific disruption of *FoxA2* can be induced by the injection of tamoxifen. We injected an adenovirus bearing a *Rev-Erbα* luciferase reporter into the tail vein, in order to transduce the vector exclusively to the liver (Saini et al., 2013). The animals were kept on a skeleton photoperiod with free access to food and water. In order to compare the effects of knocking out *FoxA2* in individual animals, we have recorded the expression of the *Rev-Erbα* reporter for about one week before the Cre-recombinase was induced by tamoxifen treatment. After the induction of Cre-mediated recombination in the liver, the luciferase signal decreased over time while the phase – the peak hours of *Rev-Erbα* luciferase expression (i.e. the phase) – remained similar (figure 7C showed a representative recording). The total locomotors activity was not affected (data not shown). Since locomotors activity cycles are a direct output of the SCN, these findings indicate that the circadian timing system in the SCN was not impaired by the liver-specific knockout of *FoxA2*, as expected. We have observed decrease of *Rev-Erbα* reporter *in vivo* with other methods of knockout *FoxA2* including tail vein injection of adenovirus expressing cre-recombinase (supplementary figure 6).

Knockout of *FoxA2* affects endogenous circadian genes

In order to examine whether the expression of endogenous circadian clock genes was also down-regulated, liver RNAs from wild-type and *FoxA2* knockout animals were collected at the zenith (ZT6) and nadir times (ZT18) of *Rev-Erbα* expression . As judged by RT-qPCR experiments only *Rev-Erbα*, *Dbp* and *Per2* mRNAs showed significant down-regulation at ZT6, while other *Bmal1* and *Clock* mRNA accumulation seemed to be less affected (figure 8A-E). Since FOXA2 can act as a pioneer factor to open the chromatin (Cirillo et al., 2002), we hypothesized that the chromatin was more compact and thus the expression of *Rev-Erbα* was decreased in the knockout animals. We tested this idea by ChIP-qPCR to detect the presence of FOXA2, RNA polymerase II (PolII), BMAL1 and histone H3 in chromatin of wild-type and knockout animals surrounding the *Rev-Erbα* promoter at ZT6 and ZT18. Figure 9A showed the location of the probes used in the ChIP-qPCR. There is a significant decrease in FOXA2 binding in the promoter region at both ZT6 and ZT18 in the *FoxA2* knockout animals, but no significant FOXA2 binding in the exon 1 and

intron 1 of *Rev-Erbα* either in the wild-type or knockout animals. However, the binding of FOXA2 in the promoter region only showed slight increase in ZT18 (figure 9B). There is no observable change of binding of BMAL1 in the wild-type or *FoxA2* knockout at ZT6 and ZT18, at the promoter or intron 1. As expected, there is no circadian change of BMAL1 binding in the exon 1 (figure 9C). Therefore, it is likely that FOXA2 regulates *Rev-Erbα* through BMAL1-independent mechanisms. In *FoxA2* knockout mice the binding of PolII was decreased in both exon 1 and intron 1 at ZT6 but not ZT18 in both wild-type and *FoxA2* knockout animals (figure 9D). At the same time, as shown in figure 9E, the binding of histone H3 significantly increased in the promoter and exon 1 and slightly increased in the intron 1 of *Rev-Erbα* at ZT6 while there is no significant change at ZT18. The inverse correlations between the binding pattern of H3 and PolII may suggest the accumulation of H3 prevent the access of PolII to *Rev-Erbα* loci. Interestingly, the increase of H3 binding at ZT6 in the *FoxA2* knockout animals was not as high as the binding of H3 at ZT18 (in both wild-type and *FoxA2* knockout animals), which may explain *Rev-Erbα* expression level decrease about 50% in the *FoxA2* knockout animals but not as low as when *Rev-Erbα* is naturally repressed at ZT18. These observations are in line with previous publications, claiming that FOXA2 can act as a pioneer factor to regulate the local chromatin structure and thereby affect the accessibility of RNA polymerase II to the promoter of *Rev-Erbα.*

DISCUSSION

We applied the STAR-PROM method to screen for IETFs affecting liver gene expression. The overall efficiency of the screening method was improved, and a novel serum-responsive transcription factor, FOXA2, was identified. FOXA2 belongs to the forkhead box (FOX) transcription factor family, named after the phenotype of the *Drosophila* mutants, which showed abnormality in the head of the fly embryo (Weigel et al., 1989). There are 50 FOX protein-encoding genes in the human genome, which are further divided into 19 subgroups (FOXA to FOXX) according to their sequence homology (Lam et al., 2013). FOXA2, also called hepatic nuclear factor 3B (HNF-3B) was first found to be essential for liver gene expression in the rat (Drewes et al., 1991; Raymondjean et al., 1991). Later on it was found to be required for embryonic development (Weinstein et al., 1994), lung morphogenesis (Zhou et al.,

1997), dopaminergic neuron generation (Domanskyi et al., 2014), regulation of metabolism (Rausa et al., 2000), and pancreatic functions (Heddad Masson et al., 2014). However, the role of *FoxA2* in circadian biology was unknown. Other members of the *Fox* family have been shown to affect circadian rhythms. For instance, insulin acted through FOXO3 to regulate *Clock* transcription synchronizing the circadian clock (Chaves et al., 2014). Here we found that protease-activated receptor 1 and thrombin can activate FOXA2 *in vitro*. The expressions of core clock genes were also affected by the disruption of *FoxA2*. Hence, FOXA2 is a new link between blood-borne signals and the circadian oscillator.

Several studies highlighted the role of FOXA2 in the metabolism, in particular during fasting. In these studies, FOXA2 was modified in response to different signaling cascades. Inspired by the regulatory mechanism of *daf-16* in *Caenorhabditis elegans*, which is the mammalian *FoxO* homolog, Wolfrum and colleagues postulated that under well-fed conditions FOXA2 was phosphorylated by AKT kinase under the regulation of the insulin/PI_3 kinase pathway (Howell and Stoffel, 2009; Wolfrum et al., 2004). Under fasting conditions, FOXA2 was subsequently found to activate lipid metabolism and ketogenesis (Wolfrum et al., 2004). Also, upon stimulation by glucagon, a starvation signal, FOXA2 was acetylated and kept inside the nuclei to promote transcription (von Meyenn et al., 2013). Recently, there were more studies published on the modifications of FOXA2 and its stability. Van Gent and colleagues found additional acetylation sites on FOXA2 by mass spectrometry analysis. They suggested that SIRT1 deacetylated FOXA2 and promoted its degradation (van Gent et al., 2014). Furthermore, SUMOylation was also detected on FOXA2, which appeared to regulate its metabolic stability (Belaguli et al., 2012).

Our experiments support the notion that modifications on FOXA2 change its transcription activating potential. The EMSA data suggested that FOXA2 can binds to DNA in un-induced cells (figure 2A). We have used different antiFOXA2 antibodies for Western blotting to show that the levels of FOXA2 in the liver nuclear extracts were nearly constant around the clock (Supplementary figure 5). This is consistent with the finding from Fang and colleagues' study that FOXA motif was constitutively bound to enhancer elements *in vivo* (Fang et al., 2014). However, it remains an open question if there are posttranslational modifications of FOXA2, which display diurnal

changes, and/or which are necessary for the activation of the protein in response to blood-borne signals. FOXA2 was shown to cooperate with other transcription factors. Zhang and colleagues showed that in response to fasting FOXA2 promoted the binding of CREB and glucocorticoid receptor to their binding sites (Zhang et al., 2005). Also, FOXA2 was found to cross talk with the pregnane X receptor in regulation of lipid metabolisms under starvation (Nakamura et al., 2007).

Various reports suggested a deleterious effect of *FoxA2* deficiency in the liver, for example, an increased accumulation of bile salts and an elevated ER stress response (Bochkis et al., 2008). The accumulation of bile salts could also induce liver inflammation and in the long-term trigger aging-related obesity (Bochkis et al., 2013). However, we did not observe significant activation of the ER stress response or inflammation in our conditional *FoxA2* mice. The Cre-recombinase used by Bochis and colleagues was activated early in development, while the Cre-recombinase used in our study was only activated after administering tamoxifen. Hence, it is possible that ER stress and inflammation phenotypes are long-term effects of *FoxA2* depletion. In contrast, we found a significant attenuation of circadian gene expression in mice with short-term depletion of *FoxA2* in the liver and in differentiated HepaRG cells treated with *FoxA2* siRNAs. Therefore, it is unlikely that ER stress and inflammation are the major causes for the attenuation circadian gene expression.

Structural analysis of FOXA2 suggested that its DNA-recognition domain resembled that of histone H5 (Clark et al., 1993). Follow-up studies showed that FOXA2 could bind and open the chromatin to facilitate the binding of other transcription factors. Therefore, FOXA2 has been classified as a pioneer factor (Cirillo et al., 2002). For instance, in ES cells FOXA2 and H2A.Z are important for nucleosome depletion during differentiation (Li et al., 2012). Moreover, in aged animals, the distribution of nucleosomes at transcription start sites was changed, and FOXA2 was proposed to be one of the factors mediating this effect (Bochkis et al., 2014). We speculated that the gradual decrease of *Rev-Erbα* expression observed in the tamoxifen-induced *FoxA2* knockout mice was a result of changes in the chromatin structure. Our preliminary results on the PolII and histone H3 binding at the *Rev-Erbα* locus supported the idea that FOXA2 might serve as modulator of chromatin structure

(figure 9A-D). Genome-wide comparison of PolII occupancy and histone nucleosome density (e.g. by measuring local histone H3 concentration) in wild-type and *FoxA2* knockout mice around the clock would provide more information on how FOXA2 affects chromatin structure and circadian gene expression. Interestingly, the circadian transcription factor CLOCK-BMAL1 heterodimer was assigned a function as a pioneer-like factor as well (Menet et al., 2014). However, it seems that the activity of CLOCK-BMAL1 was not sufficient to compensate for the lack of FOXA2 binding to the nearby region.

The activation of FOXA2 in differentiated HepaRG cells could be mediated by a whole variety of signaling cascades (figure 5). One of them may involve the protease-activated receptor 1 (PAR1) (Vu et al., 1991). This finding prompted us to analyze the effect of thrombin on FOXA2. Thrombin is a serine protease rapidly activated by the coagulation cascade (Coughlin, 2000). A class of G-protein coupled receptor is called protease-activated receptor (PAR). The ligands of PAR are part of the extracellular domain of the receptor. The proteolytic cleavage by thrombin releases this domain and thereby generates the ligand for the receptor. Downstream events after the activation of PAR included the increase in the intracellular concentration of inositol triphosphate, which activates the PI_3 kinase and increases the intracellular Ca^{2+} concentration. Upon activation, PAR1 becomes rapidly internalized and degraded in lysosomes (Ishii et al., 1993). Consequently, thrombin signaling is very time-restricted and yielded only a transient activation of FOXA2 in the differentiated HepaRG cells. Since the primary function of thrombin is in blood coagulation, it remains to be determined whether and how thrombin signaling regulates the circadian oscillator *in vivo*. However, as mentioned above, multiple signaling cascades could activate FOXA2, and our data suggested that in addition to FOXA2, thrombin signaling activated a whole variety of transcription factors, including SRF.

In conclusion, using a modified STAR-PROM screen, we have identified signaling pathways affecting the circadian clock in hepatocytes by FOXA2. FOXA2 was activated by diurnal blood-borne factors, including thrombin. Without *FoxA2*, the circadian clock dampened *in vitro* and *in vivo*. Hence, FOXA2 may act as an integrator of serum signals to maintain the function of the liver circadian oscillators.

Further experiments are necessary to specify the role of FOXA2 for the circadian oscillator in other cell lines and to characterize other immediate early transcription factors participating in the synchronization of hepatocyte clocks.

MATERIALS AND METHODS

Construction of the lentiviral STAR-PROM vector

pSTAR is a lentiviral vector derived from the vector pGTNLS-luc (Suter et al., 2011). The oligonucleotide 5'- GGTACCCTCTAGACCGCGGACGCGTCTGCAGATATC-3' was inserted into the *KpnI* and *EcoRV* restriction sites of pGTNLS-luc. The minimal promoter with the multiple cloning site (MCS) was amplified by PCR from pGL4.24 (Promega, Madison, WI) using the primers 5'-AAAACCCGGGGGCCTAA CTGGCCGGTACCT-3' and 5'-AAAACCATGGTTGTGGCCATGGTGGCTTTACC AACAGTAC-3'. The PCR product was inserted into the *KpnI* and *BstXI* restriction sites of the modified pGTNLS-luc. The resulting construct was called pSTAR with *MluI* and *NheI* restriction sites upstream of the minimal promoter for cloning (supplementary figure 1).

Rolling circle amplification reactions

The single-stranded circular DNA template used for rolling circle amplification reaction was formed by ligation of the single stranded linear c-oligo (5'-P-CAGAGCCA-N_{68}-GAAGGCTG-3') (Wang et al., 2005). The 5' and 3' ends of the c-oligo were brought into proximity by the splint (5'- TGGCTCTGCAGCCTTC-3'). The ligation was performed overnight at 4^{o}C. The ligation product was purified by phenol:chloroform extraction and ethanol precipitation. The remaining linear species were degraded by Exounclease III and VII (New England Biolabs, Ipswich, MA). The rolling circle reaction was carried out at 55^{o}C for 1.5 hours with the following ingredients: In a 50 μl reaction, 5 mg of circular DNA template, 1.2 μM primers (5'-ATATGCTAGCGCTGCAGAGCCA-3' and 5'-ATATACGCGTTCTGCAGCCTTC-3'), 6.4 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 6% DMSO, 4 ng T4 gene 32 protein (New England Biolabs, Ipswich, MA), and 300 nM dNTP in 1x ThermoPro buffer. The final reaction product was loaded onto a 1.5% agarose gel, the bands corresponding to 7-8 repeats were excised, and the DNA was extracted from the agarose using Qiagen gel purification kit (supplementary figure 2).

Construction of the STAR-PROM library

The purified tandem repeats of random sequences and the pSTAR vector were digested with *NheI* and *MluI* overnight. Both vector and insert DNAs were gel purified and ligated overnight at 16^{o}C. The ligation product was transformed into *E. coli Stbl4* by electroporation and plated onto LB plates with ampicillin (100 μg/ml). The colonies were picked into 96-well plates with 100 μl LB with ampicillin. The clones were screened by PCR with primers flanking the MCS of the pSTAR vector. In total, 35 96-well plates of colonies were screened. The cloning efficiency was around 92%. The positive clones were re-arrayed into new 96-well plates with positive and negative control clones for glycerol stocks and plasmid DNA preparations (supplementary figure 3A).

Lentiviral vector production

Lentiviral vectors from the STAR-PROM library were produced in 96-well plates. In each well, 2 x 10^4 293T cells were seeded the day before transfection. In each well, 150 ng pSTAR-PROM library DNA were mixed with 100 ng of psPAX2, 10 ng of pMD2G, 0.7 μl of XtremeGene 9 transfection reagent (Roche, Basel, Switzerland), and filled up to 20 μl with Opti-MEM medium (Thermo Fisher Scientific, Waltham, MA). The DNA-transfection agent complex was allowed to form for 30 min at RT and then added drop-wise onto the 293T cells. One day after the transfection, the medium was removed and replaced by 170 μl harvesting medium (DMEM with 10% BSA). About 48 hours after the transfection, the medium containing the lentiviral vectors of the STAR-PROM library were harvested. The medium was filtered through 0.45 μm Durapore PVDF MultiScreen filter plates (Merck Millipore, Billerica, MA) to remove any cell debris. The viruses were used directly without freezing or other concentration processes.

Lentivirus infection

On the day of virus infection, the HepaRG cells were trypsinized and seeded into 96-well plates at the density of 2 x10^4 cells per well. 80 μl of virus was added to the cells while the cells were still in suspension. The next day, the medium containing the virus was removed and new medium was added. 2 days after, the cells were trypsinized and

transferred to black-opaque bottom 24-well plates. The cells were allowed to grow confluent for 2 days. When the cells were confluent, the screening was performed.

Primary and secondary screen

The HepaRG cells were maintained in William's E medium supplemented with 10% FBS, 50 μM hydrocortisone (Sigma-Aldrich, St. Louis, MO), insulin (Sigma-Aldrich, St. Louis, MO) and 1x Penicillin Streptomycin Glutamine (Thermo Fisher Scientific, Waltham, MA). The cells were seeded at low density and allowed to grow to confluence for 2 weeks. The HepaRG cells were then maintained in medium with 2% DMSO to promote differentiation (Cerec et al., 2007). The medium was renewed every 2-3 days. Terminally differentiated HepaRG cells were used to perform the screen. The HepaRG cells were starved in medium with 0.5% FBS for 24 hours before the serum shock. A final concentration of 1μM D-luciferin was added to each well, and the basal expression level of luciferase was measured at least 5 hours before the serum shock. 20 μl of human plasma (8 different time points from 3 male and 6 female subjects) (Gerber et al., 2013) was added to each well. There are four PMT measuring four 24-well plates in parallel. The photons emitted from each well were measured for 15 seconds, every 12 minutes. The expression profile of each clone was measured for 1 day after the addition of plasma or serum (supplementary figure 3B). For the secondary screen, stable reporter cell lines of individual positive clones were established by using the calcium phosphate precipitation method. Stable cells were selected with 10 μg/ml blasticidine S HCl for 2 weeks with the medium replaced every day.

Sequence motif search

The positive clones were sequenced with the oligo (5'-AGCAAAAAGCAGATCTTGTC-3'). The sequence of the insert 68 of nucleotides was analyzed using Patch1.0 (available online: http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi). Searches were done with the "vertebrates" database. In "additional parameters", the minimal length of sites was set to 6, and the maximum number of mismatches was set to 2, and the mismatch penalty set to 100 and the lower score boundary set to 87.5.

siRNA and drug treatment

siRNA for human FOXA1 (L-010319), FOXA2 (L-010089) and FOXA3 (L-008863) were purchased from GE Dharmacon (Lafayette, CO) (SMARTpool: ON-TARGETplus). The siRNA were transfected into the HepaRG x p11D7 reporter cell line using the XtremeGene 9 transfection reagent (Roche, Basel, Switzerland). One day after the transfection, the medium was renewed. The luciferase assay was done three days after the siRNA transfection. RNA and protein samples were collected one day after the serum shock for analysis. The use of drugs to affect specific signaling pathways has been described (Gerber et al., 2013).

RNA purification and RT-PCR

Cells were washed with 1x PBS before the cells were homogenized in 1 ml of the Trizol reagent (Thermo Fisher Scientific, Waltham, MA). The subsequent RNA purification procedures were performed according to the manufacturer's protocol. The purified RNA samples were dissolved in RNase-free water. The concentrations and quality of the RNA samples were determined by the measurement of OD at A260/280. 0.5 μg of each RNA sample was used for a reverse transcription reaction for cDNA synthesis. Random hexamers and SuperScript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA) were used according to the provider's instructions. The cDNA samples were diluted before the real-time PCR. The SYBR Green Master Mix was used for RT-PCR with a lightcycler 480 machine (Roche, Basel, Switzerland), and the data were analyzed according to the ΔΔ-CT method (Livak and Schmittgen, 2001).

Protein purification, Western blots and ChIP

For samples from cell culture cells, the nuclear and cytoplasmic extracts were prepared according to (Sambrook, 2000). Nuclear extracts from mouse livers were prepared according to (Lavery and Schibler, 1993). The protein concentration was measured using the Bradford reagent. The proteins were separated on 10% SDS-PAGE, and then transferred onto nitrocellulose membranes by standard protocols. Antibodies against FOXA2 were purchased from Abcam (Cambridge, UK) and Cell Signaling Technologies (Danvers, MA). The U2AF antibody was from Sigma-Aldrich (St. Louis, MO). The PolII and histone H3 antibodies were from Abcam (Cambridge,

UK) (ChIP grade). Antibodies against clock proteins have been described (Preitner et al., 2002). ChIP experiments were performed according to (Stratmann et al., 2010) with the probes for *Rev-Erbα* described in this reference.

EMSA

10 μM each of sense and antisense oligonucleotides derived from the sequences shown in figure 2D were adjusted to 0.1M NaCl. The mixture was incubated at 95°C for 5 min, and then cooled down to room temperature gradually. The annealed oligonucleotides were labeled with α-^{32}P-dATP using Klenow DNA polymerase (New England Biolabs, Ipswich, MA). The unincorporated nucleotides were removed using a G-50 column. The protein-DNA binding reactions contained 25 mM HEPES x KOH, pH7.6, 150 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10 μg nuclear extract from the HepaRG cells, 0.2 μg/ml salmon sperm DNA, 500 ng/μl poly(dI/dC) and about 3 nM of radioactive probe. The protein-DNA reaction mix was incubated at room temperature for 5 min. The protein-DNA reaction mix was separated on a 2.4% polyacrylamide gel in 0.25 x TBE buffer. The gel was dried onto a Whatman 3MM paper (80°C for 2 hours) and then exposed to an X-ray film for autoradiography.

Animal experiments and the preparation of serum and plasma samples

All sera and plasma sample preparations were performed as described in (Gerber et al, 2013). The floxed *FoxA2* mice were obtained from the Jackson Lab (Bar Harbor, ME). The *FoxA2* mice were crossed with hairless SKH1 mice expressing a tamoxifen-inducible Albumin-cre recombinase to generate triple mutant mice. The animals were entrained to a 12 h/12 h light/dark regiment for at least 2 weeks before they were used for experiments. Adenoviral vectors containing the *Rev-Erbα*-luc reporter were injected into the tail vein of the mouse. The bioluminescence of individual mice was recorded in real time in a RT-Biolumicorder for at least one week (Saini et al., 2013). Then the mice were injected intraperitoneally with tamoxifen in corn oil or corn oil alone (control) for 5 consecutive days to induce Cre-recombinase expression. The D-luciferin was provided in the drinking water as described (Saini et al., 2013). Figure 7 and supplementary figure 6 were generated by the software, R. The code is available in the supplementary information.

Statistical analysis

All statistical analysis were performed by using ANOVA in the software Prism 6 with alpha value set at 0.05 (* represent P value < 0.5 ; ** represent P < 0.01; *** represent P < 0.001 and **** represent P < 0.0001 in all the figures.)

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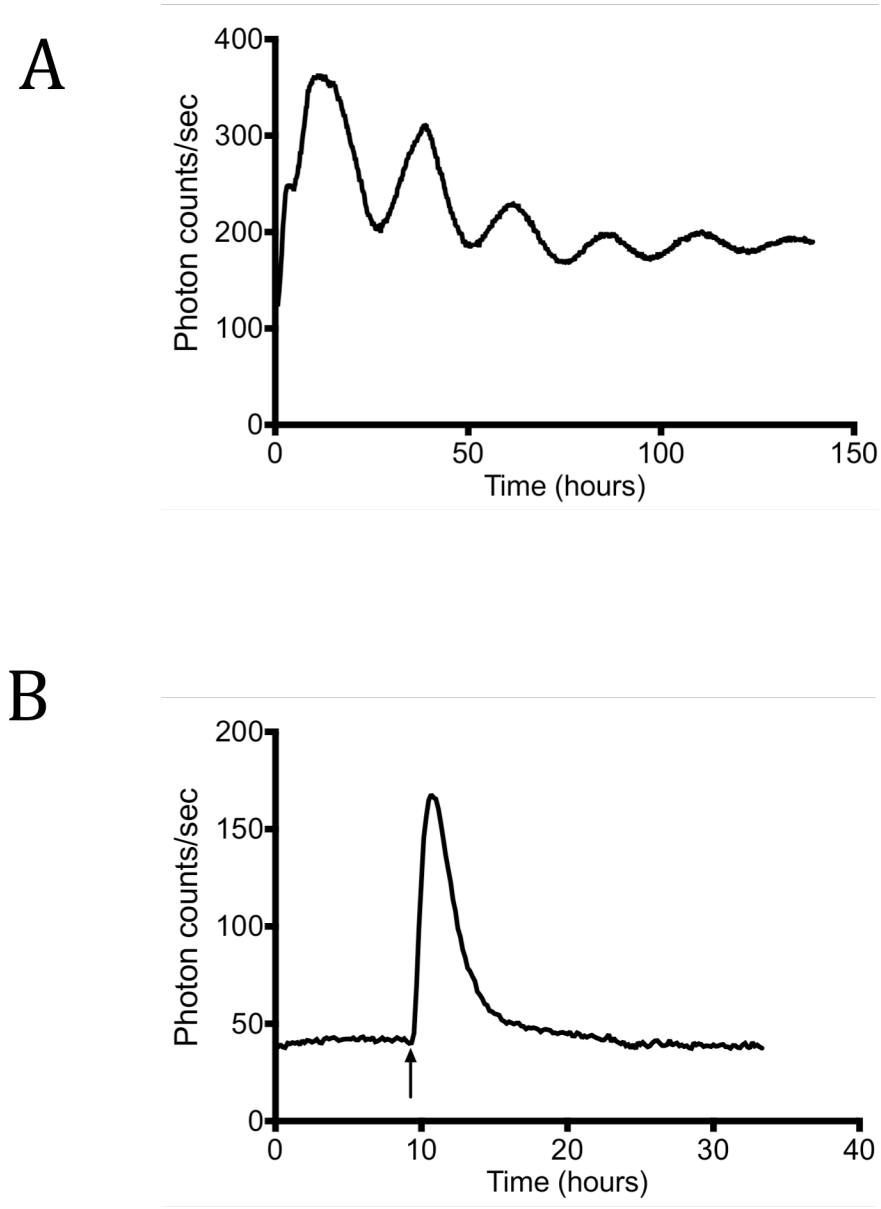


Figure 1 Characterization of differentiated HepaRG cells. Stably-transduced HepaRG cells were differentiated for 6 days with 2% DMSO and then bioluminescence was recorded as photons per sec. A) HepaRG-Bmal1luc cells display rhythmic luciferase expression for up to five days after synchronization by a serum shock. B) HepaRG-SRF cells were starved for 24 hours in serum free medium before being exposed to serum shock (arrow).

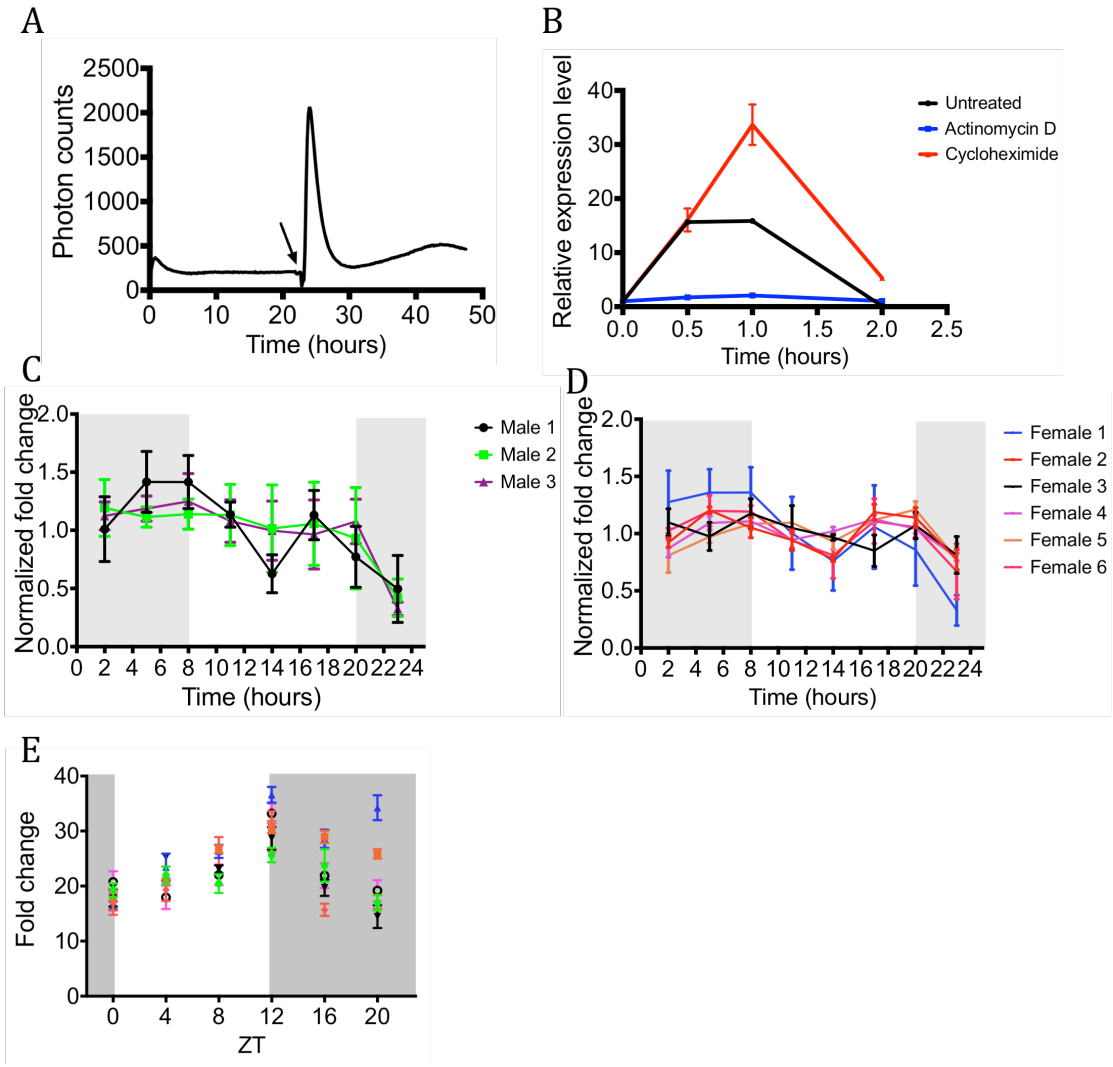


Figure 2 Clone p11D7 is an immediate early clone responding to a serum shock. A) Induction of luciferase activity of clone p11D7 after serum shock (time of the serum shock as indicated by the arrow). B) Quantification of the RNA of HepaRG-p11D7 luciferase expression treated with actinomycin D and cycloheximide. C) Induction of HepaRG-p11D7 cell line with human male plasma around the clock. D) Induction of HepaRG-p11D7 cell line with human female plasma around the clock. E) Induction of HepaRG-p11D7 reporter cells with rat serum samples obtained at 4 hours interval around the clock (n = 6 for each time point).

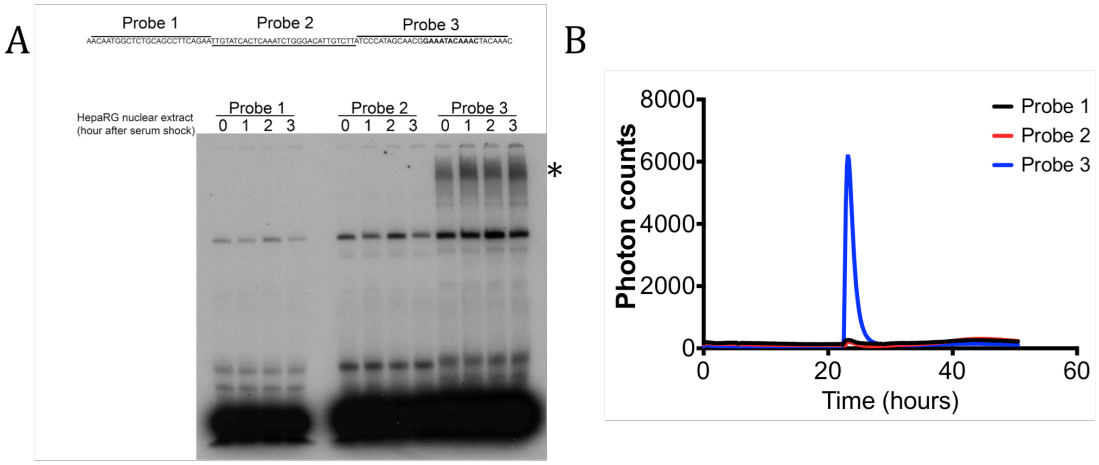


Figure 3 Identification of FOXA2 motif in the clone p11D7. A) EMSA experiment with nuclear extracts from HepaRG cells 0, 1, 2, and 3 hours after the serum shock and the DNA probes indicated on the top. B) Serum response of a reporter cell line in which luciferase expression is driven from a promoter encompassing 8 repeats of the EMSA probe 3 sequence.

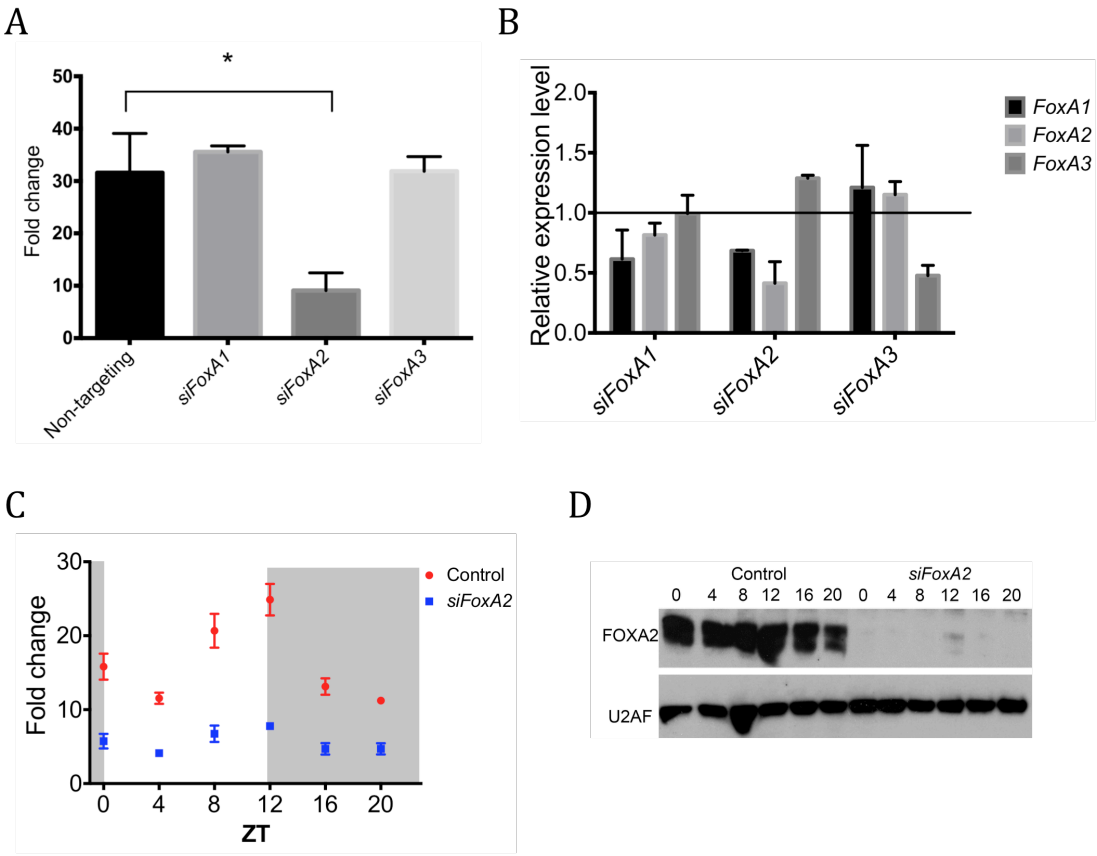


Figure 4 Identification of the serum-responsive transcription factor as FOXA2. A) Knockdown of *FoxA2,* but not *FoxA1* or *FoxA3,* reduced the induction of luciferase activity of HepaRG-p11D7 cells by serum. B) Efficiency of the siRNA-mediated *FoxA2* mRNA knockdown in HepaRG-p11D7 cells, as measured by RT-qPCR. C) The knockdown of *FoxA2* reduces the diurnal response of the HepaRG-p11D7 cells to serum. D) Western blot analysis showing the efficiency of the knockdown on *FoxA2* accumulation in nuclear extracts. U2AF was used as a loading control.

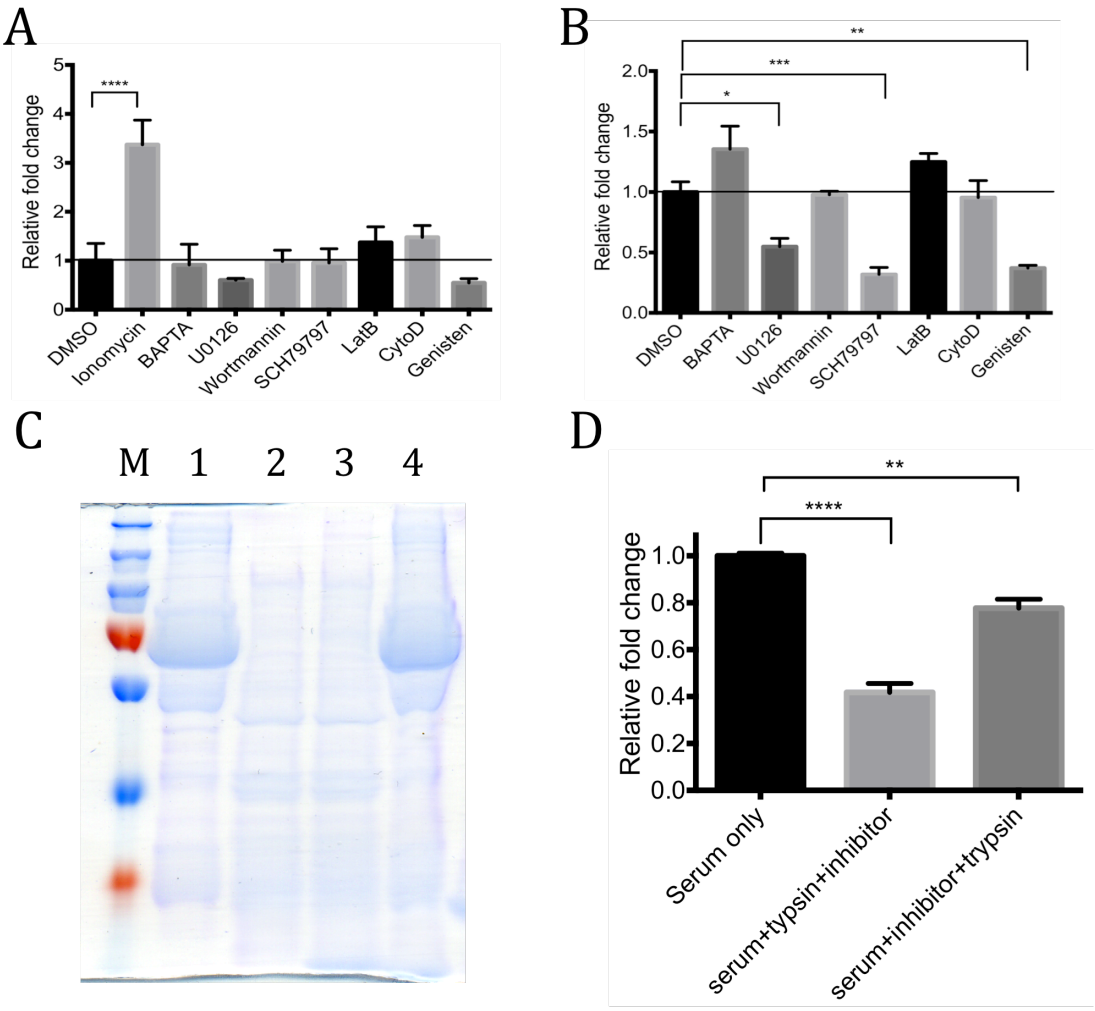


Figure 5 Characterization of signaling pathways inducing the HepaRG-p11D7 cell line. A) Quantification of bioluminescence recordings (values for photon counts measured at the peak of induction) after the treatment of HepaRG-p11D7 cells with the indicated drugs. Only the increase of intracellular Ca^{2+} by ionomycin significantly induced luciferase activity. B) Relative peak bioluminescence levels determined for HepaRG-p11D7 cells after 1-hour pre-treatment of HepaRG-p11D7 cells with the indicated drugs before the serum shock. The inhibition of the MEK kinase activity (U0126), protease-activated receptor (SCH79797), and receptor tyrosine kinase activity (Genistein) significantly reduced the immediate early response to serum. C) SDS-PAGE analysis of untreated serum (lane 1), serum digested with trypsin (lane 2), serum digested with trypsin and then treated with soybean trypsin inhibitor (lane 3), and serum incubated with trypsin in the presence of soybean trypsin inhibitor (lane 4). M: molecular weight marker. The proteins were stained with Coomassie blue. The prominent bands in lanes 1 and 4 correspond to serum albumin, the most abundant serum protein. D) Digestion of serum with trypsin significantly reduces the induction of luciferase by serum.

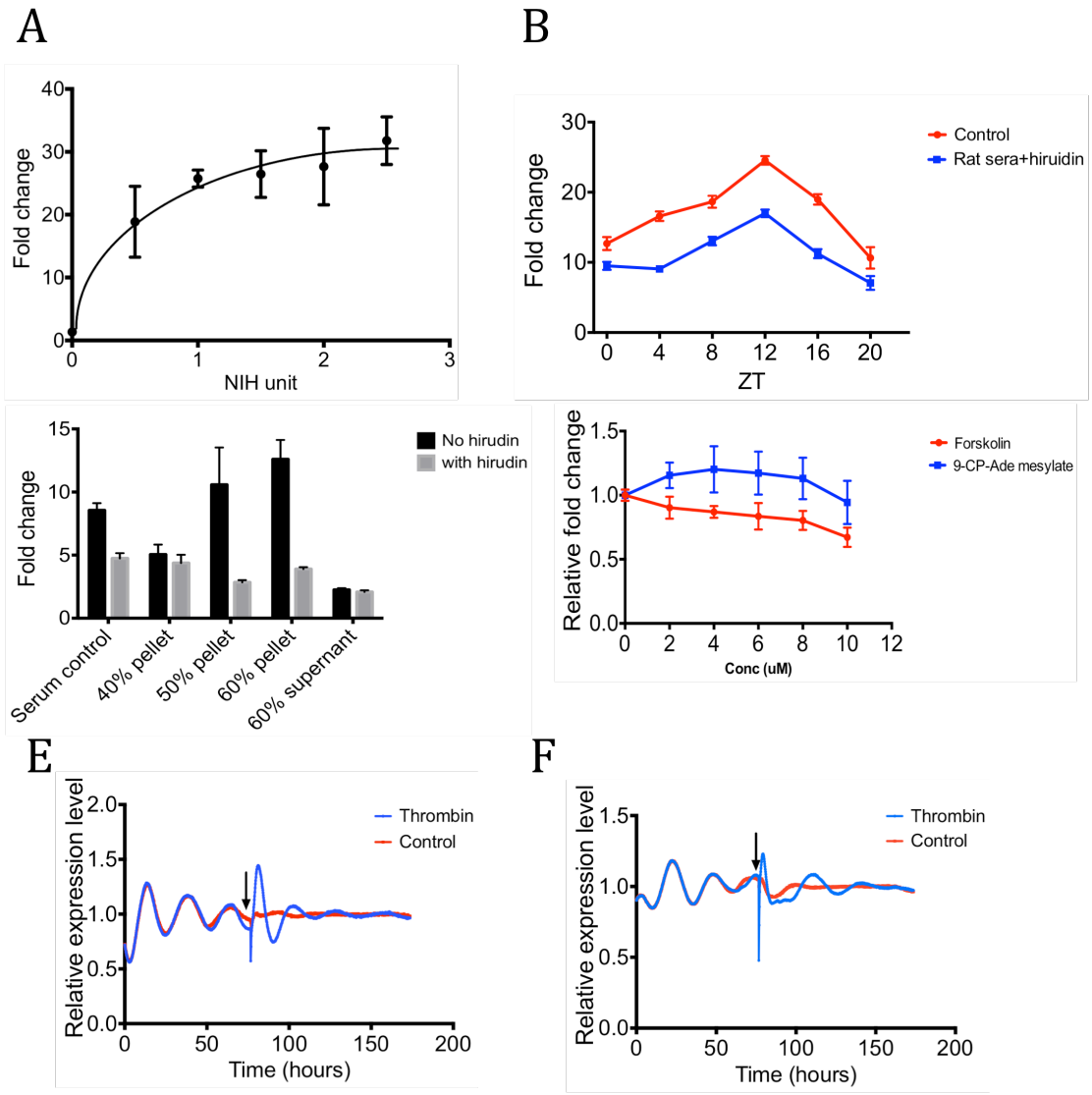


Figure 6 Identification of thrombin as an inducer of HepaRG-p11D7 cells. A) Dose-dependent induction of HepaRG-p11D7 cells with thrombin. B) The thrombin inhibitor hirudin reduces the diurnal response of HepaRG cells to serum. C) Ammonium sulfate fractionation of serum revealed the presence of thrombin activity in the precipitates obtained with $(NH_4)_2SO_4$ concentrations corresponding to 50% and 60% saturation. D) Forskolin, an inducer of cAMP signaling, reduces the response of HepaRG-p11D7 cells to thrombin, while 9-CP-Ade mesylate, an inhibitor of cAMP signaling, enhances this process (at low concentrations). E) HepaRG-Per2luc, or F) HepaRG-Bmal1luc cells were treated at the indicated time (arrow) with thrombin or control (1x PBS buffer).

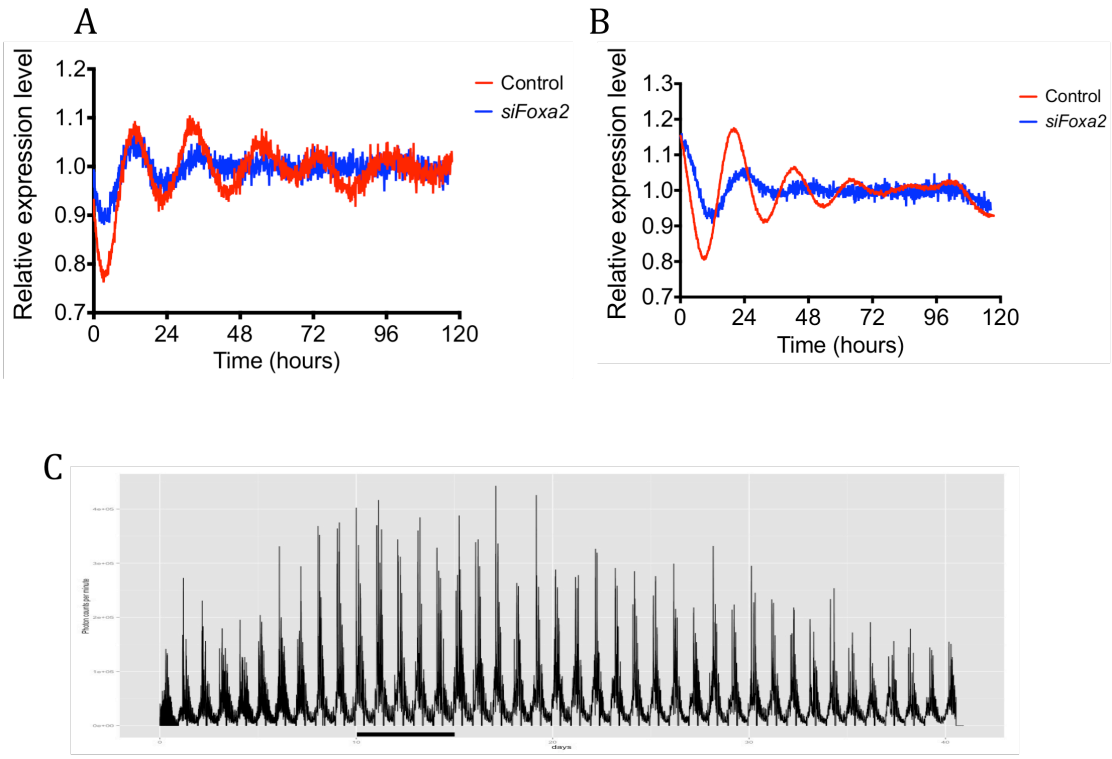


Figure 7 Dampening of circadian reporter gene expression after knockdown of *FoxA2*. The amplitude of circadian luciferase expression is reduced after the knockdown of *FoxA2* in A) HepaRG-Per2luc B) HepaRG-Bmal1luc cells. C) *Rev-Erbα* luciferase reporter activity of a liver-specific *FoxA2* knockout mouse. The black bar on the x-axis represent the 5-days with temoxifern injection.

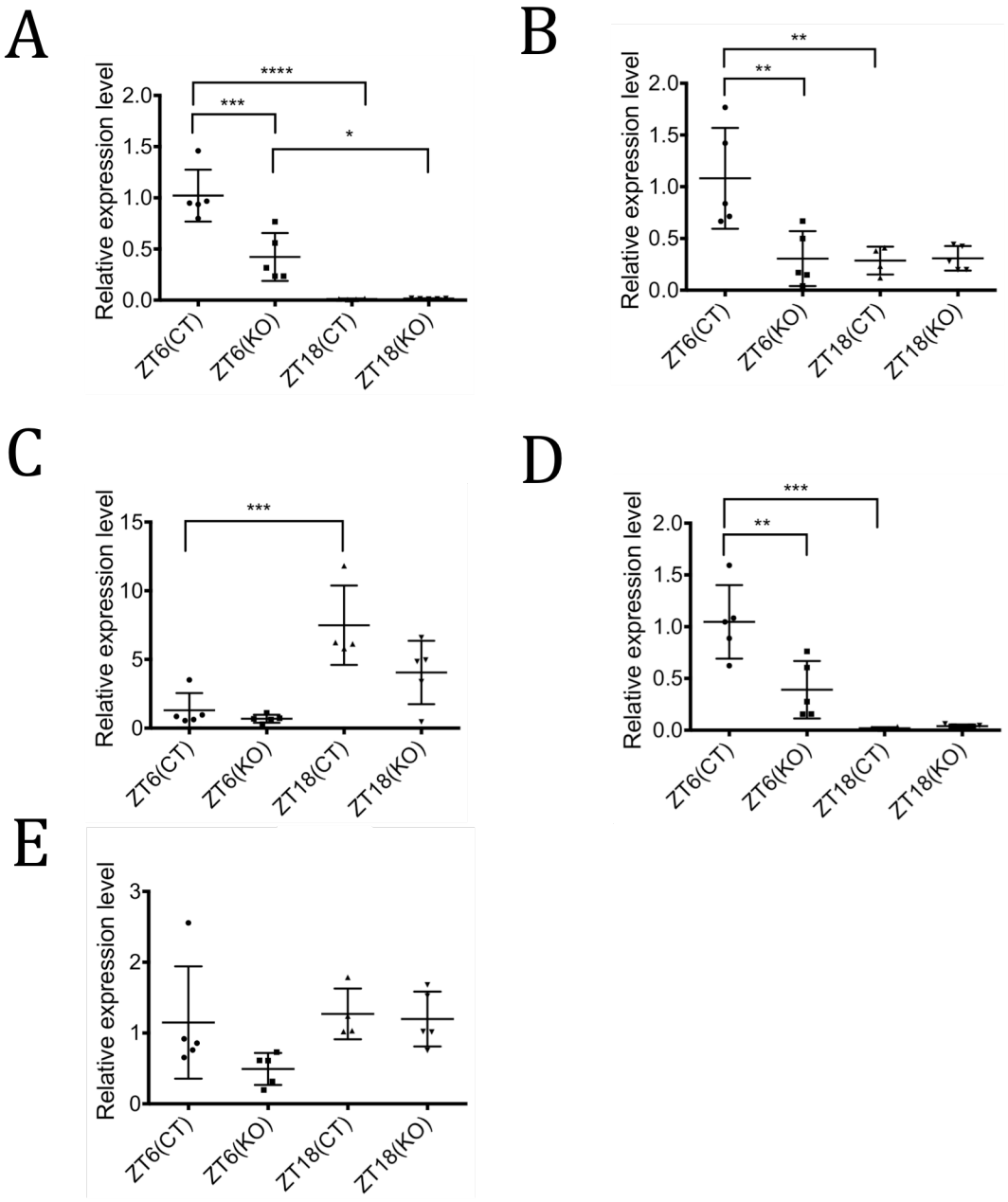


Figure 8 Analysis of the effect of the *Foxa2* knockout on endogenous gene expressions. RT-qPCR analysis of the indicated genes at two time points in control (CT) and liver-specific *FoxA2* knockout animals (KO). A) *Rev-Erbα*, B) *Per2*, C) *Bmal1*, D), *Dbp*, and E) *Clock*.

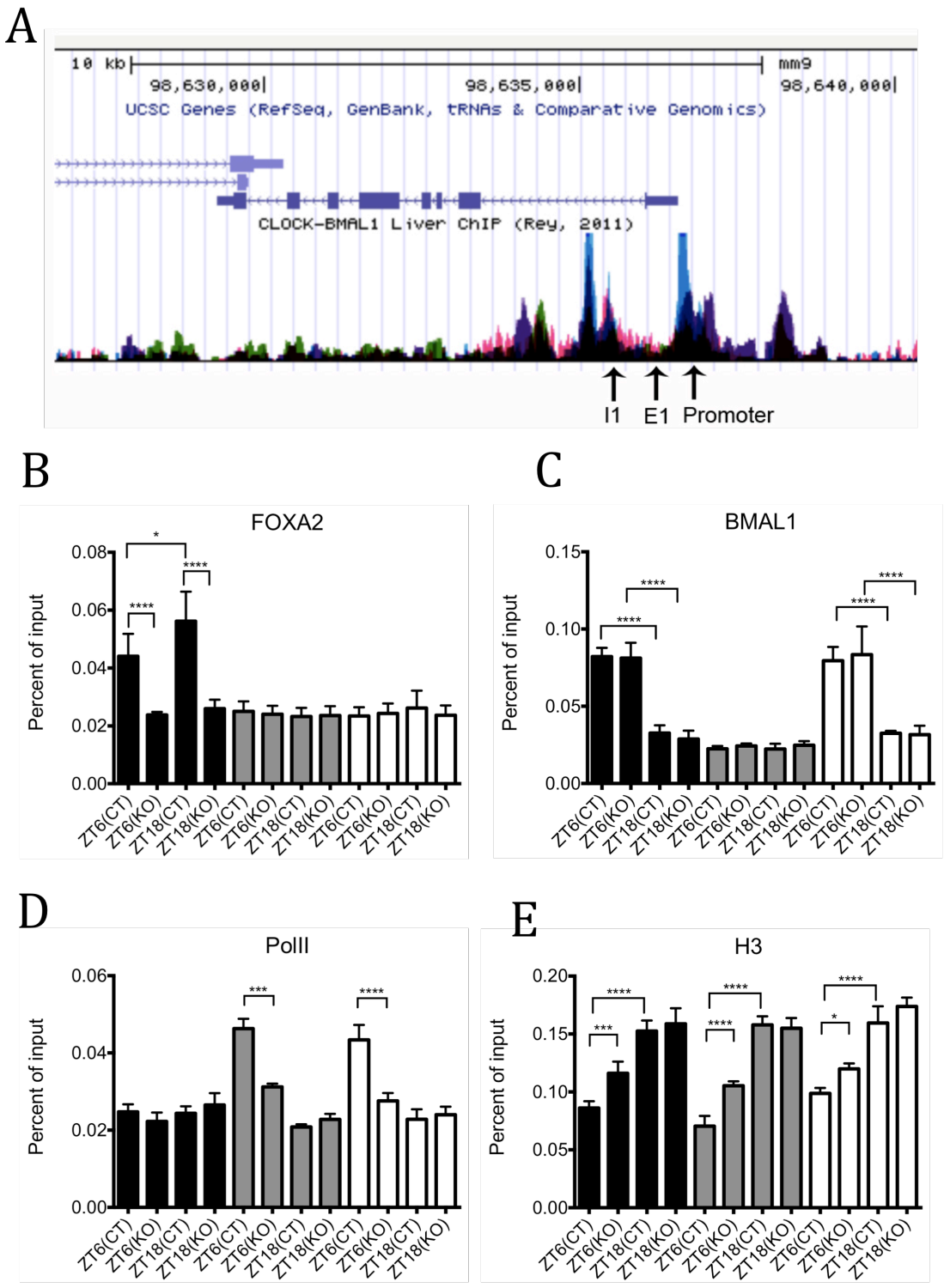
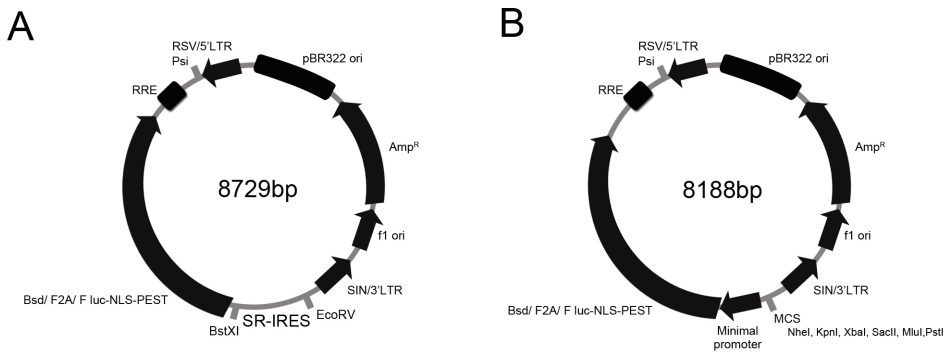
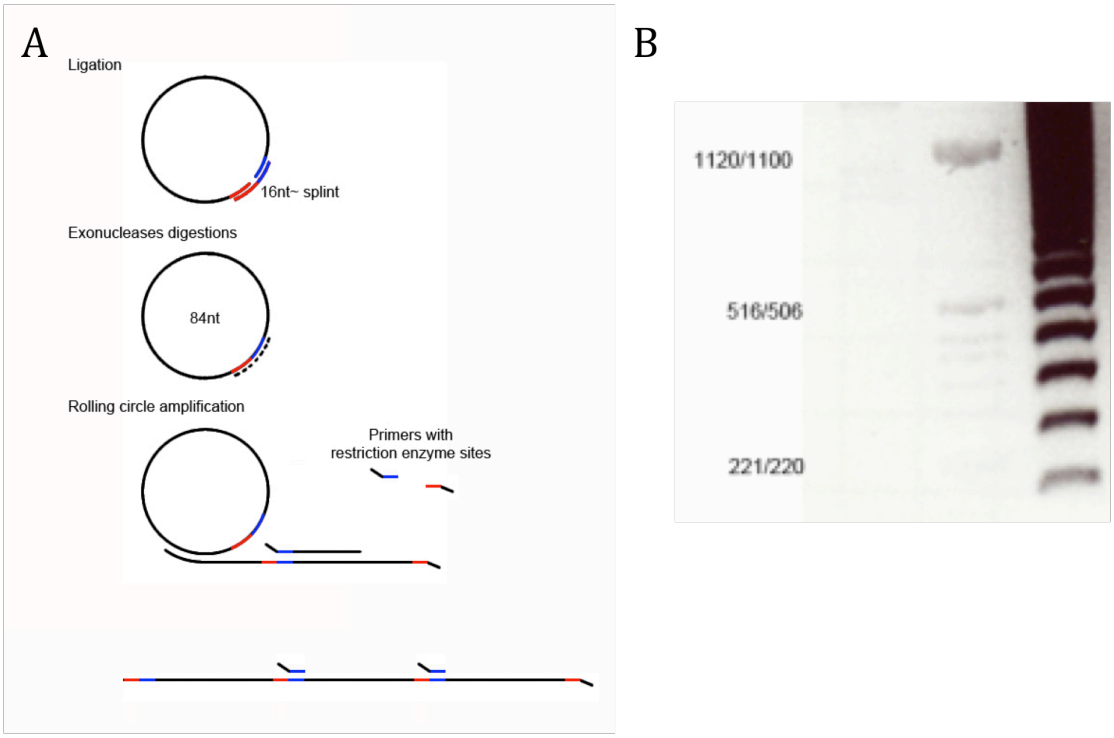


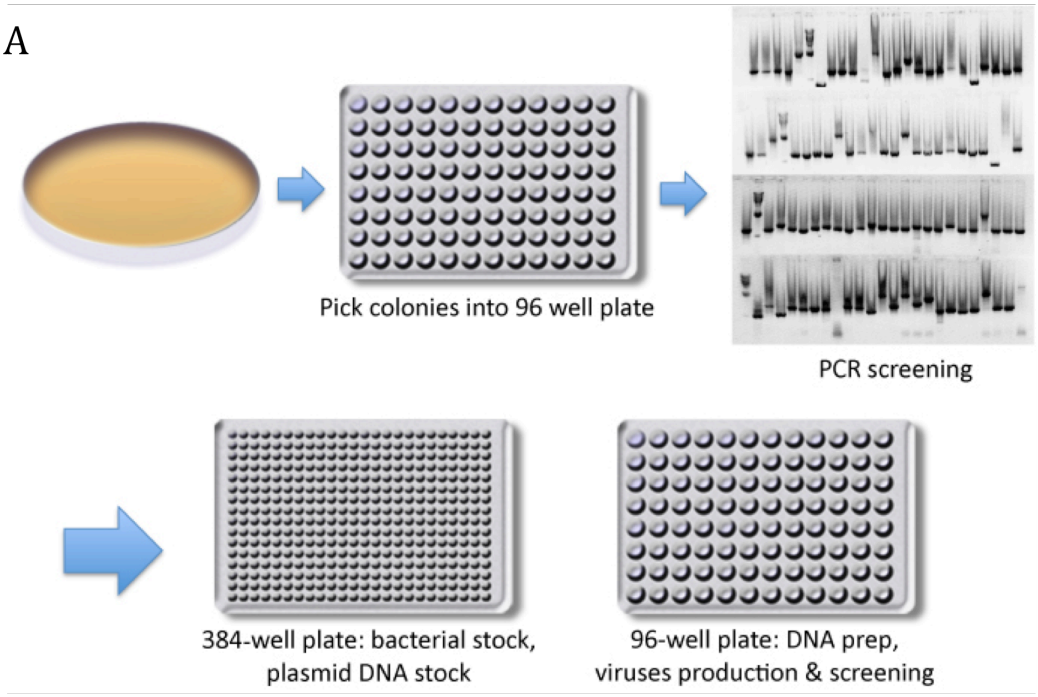
Figure 9 The effects of *FoxA2* knockdown on the binding of different factors in the *Rev-Erbα* genomic loci in the mouse livers. A) The genomic loci of *Rev-Erbα* and the probes used in the ChIP-qPCR experiments (promoter region, exon 1(E1) and intron1(I1)) ChIP-qPCR results of B) FOXA2, C) BMAL1, D) RNA polymerase II, and E) histone H3, at the promoter region (black), exon 1 (grey), and intron 1 (white) of *Rev-Erbα*.

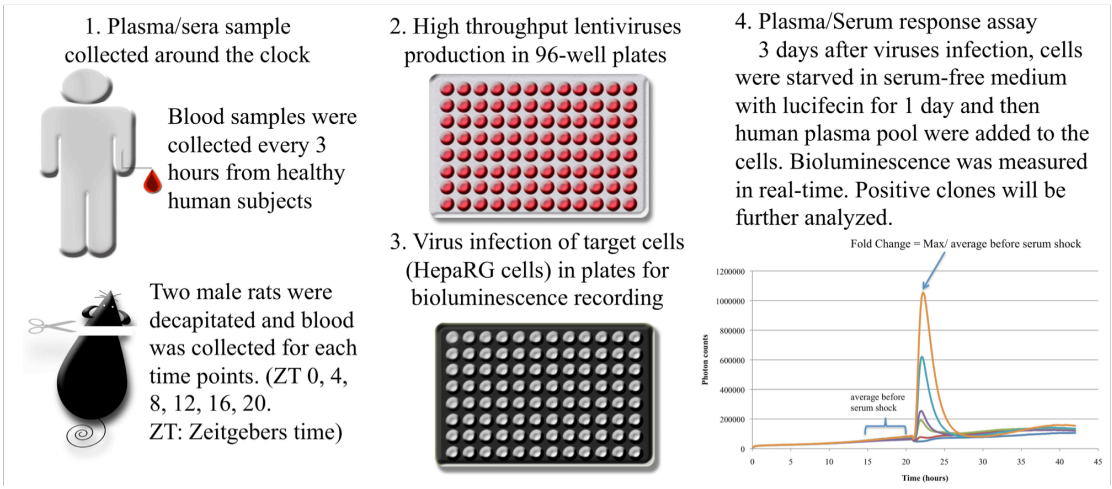


Supp. Fig. 1 The design of the pSTAR plasmid. A) Parental plasmid B) pSTAR plasmid

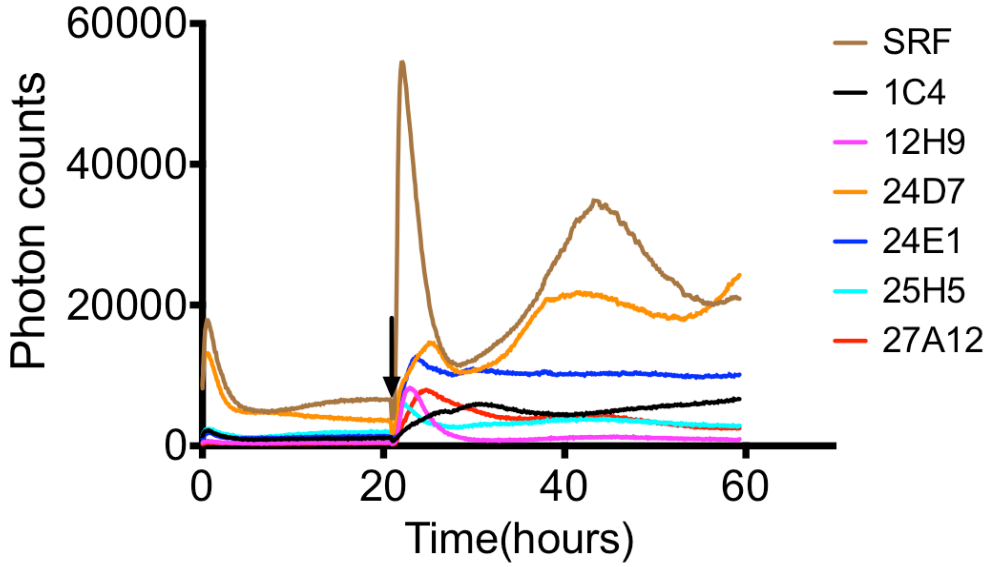


Supp. Fig. 2 Tandem repeats were generated by rolling circle amplification. A) Schematic of the rolling circle amplification reaction. Single strand circular DNA templates were generated by ligation in the present of 16nt splint. Linear DNA was digested by exonucleases. Bst DNA polymerase and the primers were added to the single strand circular DNA template to generate the tandem repeats. B) Rolling circle products were separated by 1% agarose gel. The 7-8x tandem repeats DNA fragments were cloned into pSTAR.

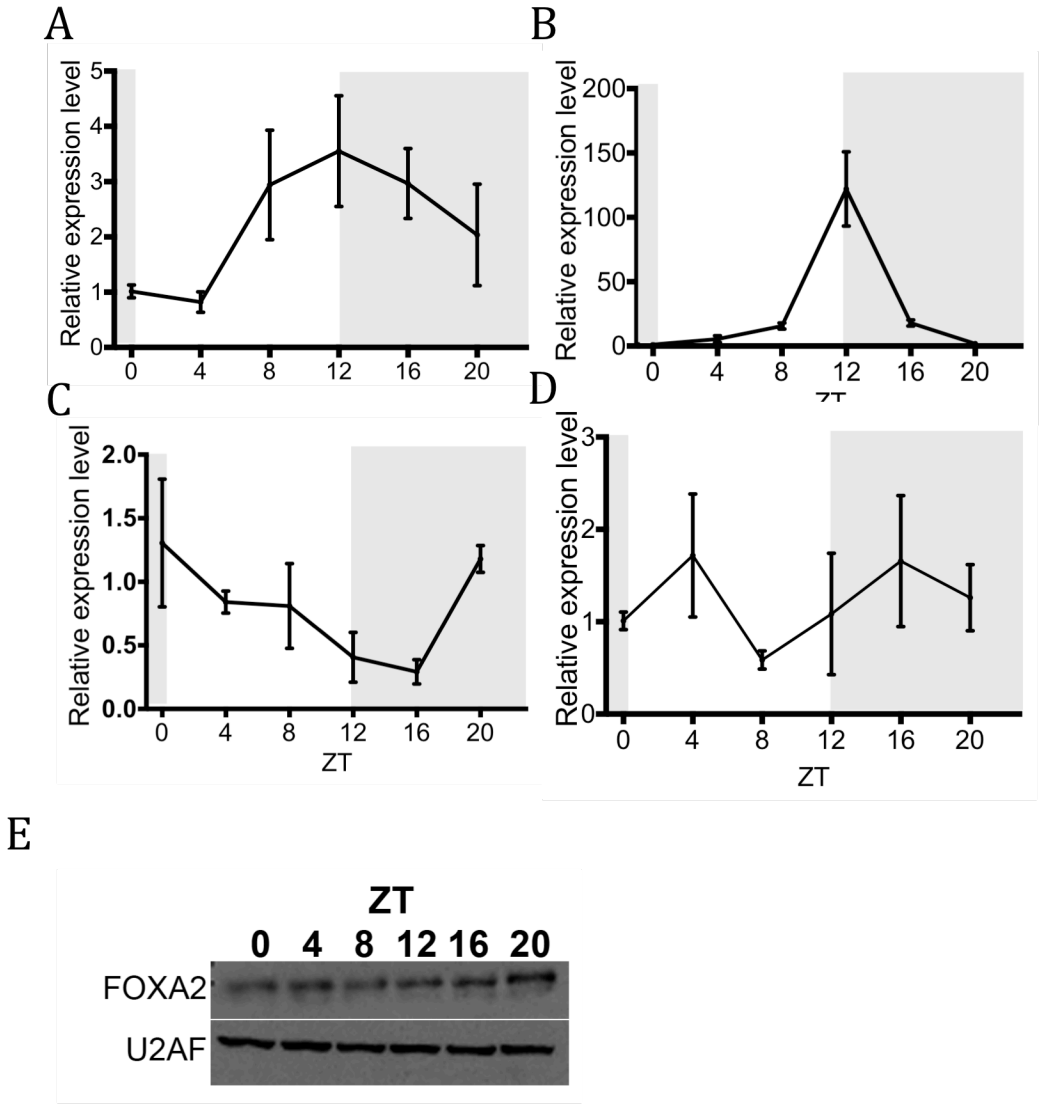




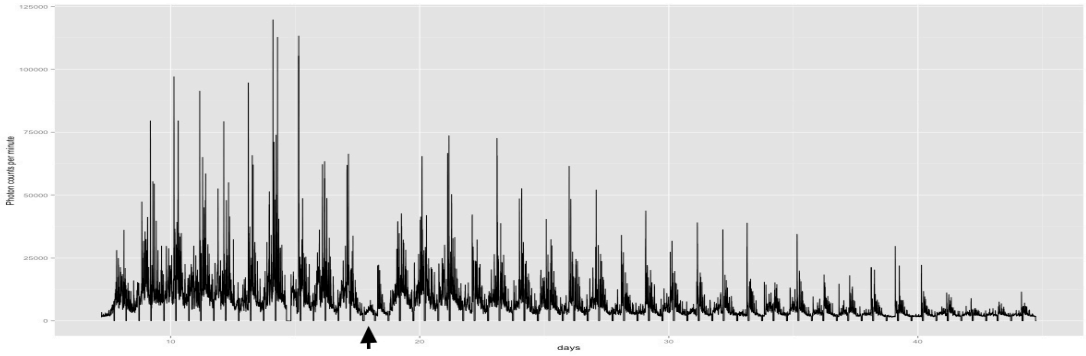
Supp. Fig. 3 Schematic diagraph of STAR-PROM screening. A) The ligation products of the pSTAR vector and tamden repeats are transformed into *E.coli*. Colonies were screened by PCR. Clones with inserts were re-array into 384-well and 96-well plates for stock and DNA preparations. Each plate contain a GFP control to monitor the lentivirus transduction efficiency and a SRF reporter as a positive control. B) Human plasma and rat sera around the clock were prepared for the screen. Lentivirus production, transduction of the HepaRGs were done in 96-well plates. Cells transduced with the lentivirus were starved for 1day in serum free-medium before the serum shock. Luciferase activities were recorded by plate reader.



Supp. Fig. 4 Thrombin has pleiotropic effects on the reporter cell lines. Shown are the measured bioluminescence counts after the treatment of a selection of transduced cells with 0.5 units of thrombin. An arrow marks the time point of thrombin treatment. Note that the positive control cell line HepaRG-SRF is responsive to thrombin as well.



Supp. Fig. 5 Temporal accumulation of mRNAs of *Per2*, *Dbp*, *Bmal1*, and *FoxA2* in livers from wild-type mice. Relative expression levels (normalized to ZT0) are shown for A) *Per2* mRNA, B) *Dbp* mRNA, C), *Bmal1* mRNA, and D) *FoxA2* mRNA. The light and dark phases are depicted as white and grey squares, respectively. E, F) Western blot of liver nuclear extracts with anti-FOXA2 antibodies (Abcam). Note that the nuclear accumulation of FOXA2 is nearly constant throughout the day. U2AF (Antibodies from Sigma)



Supp. Fig. 6 Knockout of *FoxA2* in liver by adenovirus expressing cre-recombinase. The arrow on the x-axis indicated the time when the adenovirus was injected into the animal through tail vein injection.

Supplementary information

The sequence of the synthetic promoter in clone p11D7
5'-AGAATTGTACCACTCAAATCTGGGACATTGTCTTATCCCATAGCAACG
GAAATACAAACTACAAACAA-3'

The probes used for EMSA
Probe 1:
5'- AACAATGGCTCTGCAGCCTTCAGAATTG -3'
5'- CAATTCTGAAGGCTGCAGAGCCATT -3'

Probe 2:
5'- TATCACTCAAATCTGGGACATTGTCT -3'
5'- TAAGACAATGTCCCAGATTTGAGTGA -3'

Probe 3:
5'- TCCCATAGCAACGGAAATACAAAC -3'
5'- TGTAGTTTGTATTTCCGTTGCTA -3'

The c-oligo used to generate the deletion clones of p11D7:
Deletion clone 1 (probe 1 sequence):
5'-[P]CAGAGCCAAGAATTGTATCACTCAAATCTGGGACGAAGGCTG-3'

Deletion clone 2 (probe 2 sequence):
5'-[P]CAGAGCCATCACTCAAATCTGGGACATTGTCTTAGAAGGCTG-3'

Deletion clone 3 (probe 3 sequence):
5'-[P]CAGAGCCATCCCATAGCAACGGAAATACAAACTAGAAGGCTG-3'

Primers for RT-PCR:
Human *Gapdh*:
5'- TGCACCACCAACTGCTTAGC -3'
5'- ACAGTCTTCTGGGTGGCAGTG -3'

Human *FoxA1*
5'- CCACCCGTTCTCCATCAACA -3'
5'- AGCCGTAAGGCGAGTATTGC -3'

Human *FoxA2*
5'- GCTGGGAGCGGTGAAGATG -3'
5'- ACGACGACATGTTCATGGAG -3'

Human *FoxA3*
5'- AGAACTCCATTCGCCACTCG -3'
5'- TTCCCTGAGCTGGGGTGTAG -3'

Luciferase
5'- GCAATTCACGAATCCCAACT -3'
5'- AGGTGCTTCTCGATCTGCAT -3'

mouse *Gapdh*
5’- TCAGCTCTGGGATGACCTTG -3’
5’- TGGAAAGCTGTGGCGTGAT -3’

mouse *FoxA2*
5’- ATGCTGGGAGCCGTGAAG-3’
5’- AGGATGACATGTTCATGGAG -3’

mouse *Per2*
5’- GGCAGAGCACAACCCCTCCA -3’
5’- GCGGTGGGCTCCACATCACT -3’

mouse *Rev-Erbα*
5’- GAAGTGTCTCTCCGTTGGCATGTCT -3’
5’- CGCTCTGCATCTCGGCAAGCAT -3’

mouse *Bmal1*
5’- CCAAGAAAGTATGGACACAGACAAA -3’
5’- GCATTCTTGATCCTTCCTTGGT -3’

mouse *Clock*
5’- TTGCTCCACGGGAATCCTT -3’
5’- GGAGGGAAAGTGCTCTGTTGTAG -3’

mouse *Dbp*
5’- CGCCCACCTGGTACAGAAGGA -3’
5’- TGGGCGAGTGGGTGACCAAA -3’

Primers and probes for ChIP-qPCR
m*RevErbα*_Upstream
FW: 5'- TCACATGGTACCTGCTCCAG -3'
RV: 5'- CTTTTGCCCGAGCCTTTC -3'
TM: 5'-FAM- ACAGAGGGCTCTGCGCAGGC -TAMRA-3'

m*RevErbα*_promoter
FW: 5'- ATTGCGAACTGCGGGGCTCA -3'
RV: 5'- GAGGTTGTGATGGCCTCTCTCGC -3'
TM: 5'-FAM- CGTCTCCCTCAGCCATTGCCCAGGGG -TAMRA-3'

m*RevErbα*_intron 1
FW: 5'- CCCTGACCAACCTTGAGCTA -3'
RV: 5'- CATGTCTTGCTCACCCACTG -3'
TM: 5'-FAM- AAGGTTGCCCTGCCTGGTTTAGTG -TAMRA-3'

The R code used to generate the graph of RT-Biolumicorder data

```
library(ggplot2)
graphic <- function(file){
Photocounts <- read.delim(file, header= TRUE)
Photocounts$hours <- (Photocounts$step/60)
Photocounts$days <- (Photocounts$hours/24)
p <- ggplot(Photocounts, aes(x=days, y= photon)) + geom_line()
p + labs(x="days", y="Photon counts per minute")
}

source(graphic)
graphic(“filename.txt”)
```

3. Results

Preliminary results on the identification of NR4A1 as an immediate early transcription factor affecting circadian liver gene expression

Preliminary results on the identification of NR4A1(NUR77) as an immediate early transcription factor affecting circadian liver gene expression.

ABSTRACT

The mammalian circadian timing system can be divided into a master pacemaker, residing in the brain's suprachiasmatic nucleus (SCN), and peripheral oscillators, operative in nearly all body cells. While the SCN clock is synchronized primarily by daily light-dark cycles, peripheral oscillators are phase-entrained by systemic chemical cues depending directly or indirectly on rhythms generated by the SCN master clock. Some lipid signals can penetrate the plasma membrane, bind to nuclear receptors, and thereby modulate the activity of these transcription factors. Using STAR-PROM, an unbiased screening procedure for finding binding sites for transcription factors of interest, we identified NR4A1 (also known as NUR77) as an immediate early transcription factor (IETF) responsive to blood-borne factors. The NR4A1 synthetic agonist cytosporone B mimicked the serum response. Interestingly cytosporone B decreased the expression of a *Bmal1*-luciferase circadian reporter, but not that of a *Per2*-luciferase circadian reporter in the human liver cell line, HepaRG cells. We speculated that NR4A1 may compete with members of the RAR-related orphan receptor (ROR) family for the binding to the ROREs present in the *Bmal1* promoter, but stimulates transcription less efficiently than RORs when bound to these cis-acting elements. To examine the influence of NR4A1 on the circadian clock *in vivo*, food inversion experiments were preformed to examine the kinetics of the changes of NR4A1 and core clock genes. We found that NR4A1 might be upstream of the core clock genes under food-induced phase shift conditions. To identify the potential blood-borne ligands for NR4A1, we fractionated rat serum biochemically. Removing the lipids from the serum abolished the induction of NR4A1-mediated reporter gene expression. We are focusing on the characterization of hydrophobic serum fractions and hopefully we will be able to identify the ligand of NR4A1.

INTRODUCTION

In mammals, nearly every cell harbors a self-sustained, cell autonomous circadian oscillator, relying on two interconnected negative feedback loops in core clock gene expression (Dibner et al., 2010; Partch et al., 2014). As individual cells

display considerable variations in period length of the circadian clocks, they have to be synchronized daily in order to keep phase coherence. The synchronizations are accomplished in a hierarchical fashion. The suprachiasmatic nucleus of the ventral hypothalamus is the master clock that directly synchronized by the photoperiod, so as to stay in resonance with the outside world. The SCN clock then generates rhythmic signals, either indirectly by driving rest-activity and feeding-fasting rhythms, or more directly by controlling the cyclic production and secretion of humoral and neuronal cues. These signals then regulate overt daily cycles in physiology by governing the temporal regulation of clock-controlled genes. These can be accomplished in two distinct ways: (1) the cyclic signaling cues directly control the expression of clock-controlled genes in peripheral organs, or (2) they synchronize cellular oscillators in the cells of these organs, which then drive the rhythmic expression of clock-controlled genes. Some of the involved systemic signals, for example glucocorticoid hormones, can act through both of these pathways. Otherwise put, their rhythmic accumulation in the blood can engender both the cyclic activity of glucocorticoid responsive genes (e.g. *Tyrosin aminotransferase*, *Tat*) (Harza et al., 2007) and the synchronization of peripheral clocks (Balsalobre et al., 2000; Le Minh et al., 2001). In addition to the glucocorticoid receptor, many nuclear receptors play pivotal roles in circadian biology (Zhao et al., 2014; Yang et al., 2007), and some of them, such as the members of the ROR and REV-ERB families, are even core clock components.

The major purpose of circadian oscillators is to generate transcription factors with circadian activities, which can then control the temporal expression of the clock-controlled genes modulating circadian behavior and physiology. The PAS-domain helix-loop-helix transcription factors BMAL1 and CLOCK, which form heterodimeric complexes binding to E-box motifs within promoters and enhancers of their target genes, lie at the core of molecular clocks. Their cyclic activity is driven by large repressor complexes containing the Cryptochrome proteins CRY1 and CRY2 and the Period proteins PER1 and PER2 (Kim et al., 2014; 2015). Since the *Per* and *Cry* genes are themselves among the CLOCK-BMAL1 target genes, the y downregulate their own expression, once the PER-CRY complexes have reached a critical concentration. Thereby, CLOCK-BMAL1 and PER-CRY complexes generate the canonical negative feedback loop within the molecular oscillator. A second feedback loop, connected to the canonical feedback loop, involves the competitive

binding of the orphan nuclear receptors RORα/β/γ, acting as activators of these genes, and REV-ERBα/β, acting as repressors, to RORE elements within the *Clock* and *Bmal1* genes.

While the two-coupled negative feedback loops are indispensible for the generation of circadian rhythms in the SCN (Bunger et al., 2000; DeBruyne et al., 2007) and in tissue explants and cultured cell (DeBruyne et al., 2007; Kornmann et al., 2007), the situation is different in liver (and probably in other peripheral tissues) of animals. In fact, robustly circadian PER and CRY protein expression persists in the absence of CLOCK and BMAL1 in the livers of mice with a rhythmic SCN (Lamia et al., 2008; DeBruyne et al., 2006; Kornmann et al., 2007). This strongly indicates that in intact wild-type animals, the cyclic transactivation potential of CLOCK-BMAL1 heterodimers can be driven by systemic signals. Obviously, this immediately provides a mechanism by which the countless peripheral body clocks are kept in synchrony.

Given that peripheral oscillators are driven by systemic cues, the identification of the identification of the underlying signaling pathways is of major importance for the understanding of the mammalian circadian timing system. Using a modified STAR-PROM procedure for the unbiased identification of serum-responsive transcription factors, we identified the nuclear receptor NR4A1 as an immediate early transcription factor (IETF) influencing the phase of the liver circadian oscillator. Furthermore, we discovered that the NR4A1-copurifies with the lipid fraction of the serum.

RESULTS AND DISCUSSION

The Synthetic TAndem Repeat PROMoter (STAR-PROM) technology exploits the high statistical frequency of transcription factor binding sites in random synthetic DNA. Briefly, STAR-PROM libraries are constructed by inserting tandem repeats encompassing 68 bp of synthetic random DNA into a luciferase reporter gene upstream of a basal promoter (Gerber et al., 2013). Individual STAR-PROM plasmids are then cloned, and a sufficient number of them are transfected into cells kept in multi-well plates (one clone per well), The cells are then treated with serum (or another source of signaling molecules) and the bioluminescence is recorded in real

time using photomultiplier technology). For two reasons we modified the original STAR-PROM technology, in replacing transfection with lentiviral gene delivers. First, this method should be applicable to a large variety of cells, including “poorly transfectable primary cells. Second, the stable integration of the reporter gene allows for an increased sensitivity in detecting induced STAR-PROM vectors, since in contrast to transient transfection, in which the baseline of bioluminescence changes dramatically, this baseline is constant in stably transduced cells (Hui et al., manuscript in preparation; see Chapter 2 of this thesis).

A total of 30 serum-responsive clones were found by screening about 2800 STAR-PROM vectors with this modified method. Here, we focus on the characterization of clone p9A3 (96-well plate no. 9, A3 position) and its properties. An initial characterization by an *in silico* motif-search revealed a potential binding site for the nuclear receptor NR4A1 in the synthetic promoter elements. This orphan nuclear receptor was initially identified as a factor induced by serum growth factors (Williams and Lau, 1993). A gel mobility shift experiment (EMSA) using nuclear extracts from mouse liver at six different time points every 4 hours around the clock and 23 bp sub-fragments of the 68 bp repeat revealed that there were multiple factors binding to the insert of clone p9A3. Interestingly, one of them was binding to the DNA probe in a diurnal fashion, with a peak at ZT8 (figure 3.1). The phase of the binding pattern was slightly delayed with regard to NR4A1 mRNA accumulation, which peaks at ZT4 (see Yang, 2006).

The clone p9A3 behaved like a real immediate early responsive clone, because the accumulation of luciferase mRNA was abolished by the transcription inhibitor actinomycin B, but not the protein synthesis inhibitor cycloheximide (figure 3.2A). Knockdown of NR4A1 in the HepaRG-p9A3 cell line reduced the serum response, confirming that NR4A1 was responsible for the major part of the induction (figure 3.2B). Since the synthetic agonist cytosporone B (CsnB) (Zhan et al., 2008) is capable of activating NR4A1, we tested if CsnB could induce the clone p9A3 (figure 3.2C). The dose response curve showed a positive correlation between the increasing concentrations of CsnB added to the cells and the increasing induction of the luciferase reporter expression. The HepaRG-p11D7 reporter cell line, carrying a FOXA2 binding site reporter gene (see Part 2 of this thesis), was used as a negative

control. There was no induction of HepaRG-p11D7 reporter even with the highest amount of CsnB. These results demonstrated that the clone p9A3 was a genuine NR4A1 reporter. Next, the effect of activation of NR4A1 on the circadian clock was examined *in vitro*. CsnB was added to HepaRG-Bmal1luc and HepaRG-Per2luc cell lines (figure 3.3). The addition of CsnB caused a reduction of the luciferase signal in HepaRG-Bmal1luc cells (figure 3.3A) while HepaRG-Per2luc cells showed no reduction in amplitude, but a very slight phase shift (figure 3.3B). Hence, the effect of CsnB was rather specific for the *Bmal1* reporter whose rhythmic expression relies on the interplay of rhythmic binding of nuclear receptors. Given the sequence similarity of ROREs and NR4A1 binding sites it is possible that NR4A1, after activation by serum, competed with the binding of RORs and REV-ERBs to the RORE regulatory sites within the *Bmal1* promoter.

There are several findings suggesting a regulatory role of NR4A1 in nutrient processing. For example, overexpression of NR4A1 in liver reveals its regulation on lipid metabolisms by suppressing SREBP1c (Pols et al., 2007). Furthermore, NR4A1 is up-regulated in muscle and liver from food-restricted rats (Oita et al., 2008) and in adipose tissue during fasting (Perez-Sieira et al., 2014). As feeding-fasting rhythms are potent *Zeitgebers* for peripheral clocks, a restricted day-time feeding experiment (food inversion experiment) inverts the phase of circadian gene expression in the liver (Damiola et al., 2000). Normally the mice are kept under *ad libitum* food conditions and then the SCN oscillator governs their peripheral oscillators. Upon daytime feeding, the phase of the liver oscillator shifts gradually over the course of two weeks to fit the new phase of the feeding regimen (Stokkan et al., 2001; Damiola et al., 2000). Since NR4A1 might relay the feeding or fasting signals to the circadian clock, we performed food inversion experiments to see if the expression of NR4A1 would be affected. As shown in figure 3.4A, the phase of accumulation of the circadian oscillator components PER2 shifted in response to daytime feeding by about 12 hours. The same holds true for the daytime-dependent phosphorylation pattern of BMAL1. NR4A1 was found to accumulate in liver nuclei with a peak about ZT4 while in daytime feeding regime, it shifted to ZT16. The same observations were made for the accumulation of the corresponding mRNA accumulation (figure 3.4B) (Yang, 2006). There are two possibilities to explain our observations. First, NR4A1 could be a downstream target of the circadian oscillator. After two weeks of food inversion, the

circadian oscillator was phase-inverted, therefore the expression of NR4A1 was shifted accordingly. Second, NR4A1 relayed signals related to the new feeding regimen to the circadian clock, which would predict that the expression (and/or activity) of NR4A1 changed before the circadian clock was phase-inverted. To differentiate between these two scenarios, the kinetics of the gene expression changes was analyzed with higher temporal resolution. We restricted the food access to the light phase and analyzed the gene expression at ZT4 and ZT16 of day 2, 4, and 6 after the food inversion. The gene expression of *Rev-Erbα* remained in the same phase after 2 days of daytime-restricted feeding. At day 4 the expression of *Rev-Erbα* was similar between ZT4 and ZT16 indicating the beginning of the phase shift. After 6 days of light-time feeding, the *Rev-Erbα* expression was higher at ZT16 than ZT4, which indicated that the phase inversion had been completed (see day 1). For NR4A1, a change in expression can already be seen after 2 days of food inversion, suggesting that it immediately follows the new phase of food availability. Hence, it is conceivable that NR4A1 might function upstream of the core clock genes and drive the phase shift.

From the structure study of NURR1 (also known as NR4A2), a closely related family member of NR4A1, the authors suggested that NR4A1 belongs to a class of ligand-independent nuclear receptors (Wang Zhulun et al., 2003). Nonetheless, Vinayavekhin and Saghatelian adopted a metabolomic approach to identify potential ligands of NR4A1 *in vitro*. They purified NR4A1 and incubated it with brain and testis lipid extracts. They found unsaturated fatty acids are enriched with the NR4A1 protein (Vinayavekhin and Saghatelian, 2011). However, they didn't perform any biological assay to verify if the enriched lipids can activate the transcription activity of NR4A1. In mouse beta cell, NR4A1 can be induced by lipotoxic fatty acids (Briand et al., 2012). Obviously, it would like crucial to identify the potential ligand or activator of NR4A1 in serum. First, treatment of serum with the protease such as trypsin did not abolish the induction of HepaRG-p9A3 reporter cell line, suggesting that the active components are not of proteinaceous nature. In contrast, removing the lipids from the serum had a strong attenuating effect of NR4A1 induced gene expression (figure 3.6). This experiment suggested that the ligands were of hydrophobic nature, in line with previous publications. Conventionally, purified lipids are dissolved in 4% fatty acid-free BSA solution before applied to the cells for *in vitro*

assays. However, no induction of HepaRG-p9A3 reporter cell line was observed by dissolving the lipids fraction in fatty acid-free BSA solution or DMSO. Also, since the signal might be small enough to be separated from the serum by dialysis or a porous filter, filtration through a 3 kDa cut off filter unit was used to separate potential small molecules from the serum. However, the relevant signaling molecules stayed in the large molecular weight fraction (data not shown). These observations suggested that the bio-active lipid species might be associated with carrier proteins present in the blood. A method was developed to reconstitute the lipoprotein complex by first mixing the extracted lipids with the carrier proteins (charcoal-stripped serum) and detergent, such as sodium cholate. Detergent was then removed by an overnight dialysis (Matz and Jones, 1982). We observed the induction of HepaRG-p9A3 reporter cell line with the reconstituted lipoprotein complex (as shown in figure 3.7), suggesting that it was indeed hydrophobic compounds present in the serum inducing the activity of NR4A1.

Since the blood-borne bio-active lipid species are likely to be in a lipoprotein complex, we fractionated the serum by ultracentrifugation to separate cholesterol containing lipoproteins from other lipids containing constituents. KCl and NaCl were added to the serum to reach the density of 1.21 g/ml. The mixture was centrifuged for 20 hours at about 280,000 g-force (Redgrave et al., 1975). Lipoproteins like VLDL, LDL, HDL were enriched in the upper phase, while the lower phase should be free of cholesterol containing lipoproteins. Both cholesterol lipoprotein containing fractions and cholesterol-lipoprotein-free fractions showed induction (figure 3.8). Lipids from the cholesterol lipoprotein-free fraction were extracted and further separated into different lipid classes by solid phase separation (SPE) column (Ichihara et al., 2011). The acetone fraction contains the highest induction activity (figure 3.9), therefore it will be subjected to HPLC for further separation and identification of active lipid components (on-going work).

To conclude, we characterized NR4A1 as a new putative immediate early transcription factor participating in circadian clock regulations in responsive to lipids in the blood. However, the final chemical identification of active compounds is still outstanding. The preliminary data suggested that the lipid factors are associated with proteins in the blood, and that this association is important for its NR4A1 inducing

capability. Further identification of the compound by GS-MS and NMR are going. Nevertheless, the food inversion kinetics experiment suggested that NR4A1 may be among the candidates for directly mediating the feeding- or fasting-related signals to the circadian oscillator. If so, this may be accomplished through RORE/RREs elements within the Bmal1 promoter, which bind orphan receptors of the ROR and REV-ERB families, and, probably, NR4A1. Genome-wide ChIP-sequencing might provide valuable information on the downstream targets of NR4A1 and thereby contribute to our understanding of how this nuclear receptors modulates the circadian clocks.

MATERIALS AND METHODS

As most of the experimental protocols are similar to the previous part of the study, please refer to the chapter 2 for details about the STAR-PROM screening, cell cultures, nuclear extract preparation, western blot, siRNA, RNA extraction, RT-PCR and EMSA. The list of primers used for RT-PCR and oligos used for EMSA probes are available in the supplementary information.

siRNA, drug treatment and antibodies

siRNA for human NR4A1(L-003426) was purchased from GE Dharmacon (Lafayette, CO) (SMARTpool: ON-TARGETplus). Cytosporone B (C2997) was purchased from Sigma-Aldrich (St. Louis, MO), and dissolved in DMSO at 10mM (stock concentration). Antibodies against NR4A1 was purchased from Novus (NB100-56745). The U2AF antibody was from Sigma-Aldrich (St. Louis, MO). Antibodies against clock proteins have been described (Preitner et al., 2002).

Animal experimentations

The animals were entrained in 12/12 light-dark cycle for at lease two weeks before the experiments. For the food inversion experiments, the food was removed from ZT12 to ZT0 (subjective night) and food was given from ZT0 to ZT12 (subjective day), while the animals had free access to water throughout the day.

Serum lipids purifications

Delipidation of serum was performed with diisopropyl ether in butanol (DIPE-BU) or tert-Butyl methyl ether (MTBE) (Sigma-Aldrich, MO). 1 volume of serum were

mixed with 2 volume of organic solvents for 5 minutes. Then centrifuged for 5 min at 12,000g to separate the organic phase and aqueous phase. The serum lipids are in the organic phase which are dried under nitrogen gas and then store at -80^{o}C until used.
For lipid-free serum protein preparation, 1g of dextran-coated charcoal (Sigma-Aldrich, MO) was used for every 50ml of serum. The charcoal was mixed with the serum at 4^{o}C overnight. The charcoal was removed by centrifugation and the serum was filtered with 0.2 mm pore-size filter unit.

The reconstitution of serum lipoprotein complex was outlined as the following procedures. The dried lipids were dissolved in DMSO, and then mix with lipid-free serum proteins. Sodium cholate (3% dissolved in water as the stock) was added to the mixture at the final concentration not exceeding 0.15%. The lipid-protein-sodium cholate mixture was dialyzed with 1x PBS at 4^{o}C overnight.

The solid phase extraction was preformed by using LiChrolut Si (25-40mm) columns (Merck, Germany). The columns were washed with 10ml hexane and 6ml acetone and then dried overnight before used. Lipids from about 100ml serum were loaded into the column and then dried. The fractions were collected as the following order. 1) 3ml 1% methyl acetate in hexane (cholesterol esters); 2) 3ml of 2.5% methyl acetate in hexane (triglycerides), 3) 3ml acetone (contains less well characterized lipids); 4) 4ml methanol (glycerophospholipids). The fractions were dried under nitrogen gas and store at -80^{o}C before used.

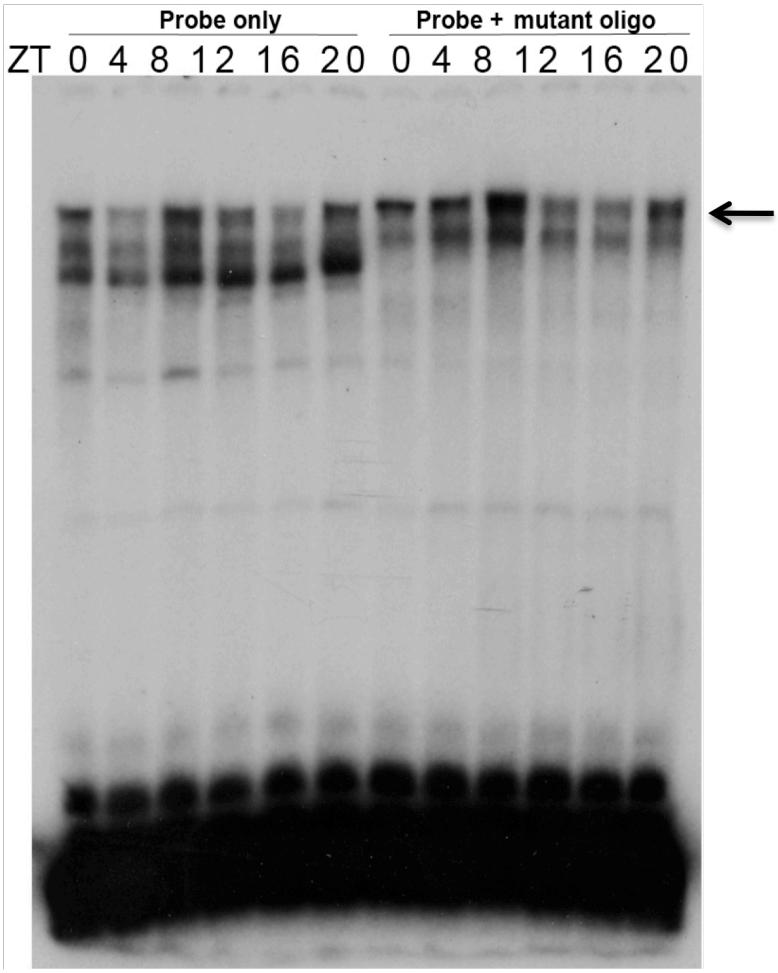


Figure 3.1 Identification of a diurnal protein:DNA complex binding to the NR4A1 element. An EMSA experiment was performed with a ^{32}P-labeled oligonuceotide containing the potential NR4A1 binding site and nuclear extracts from mouse liver harvested at the indicated time points. On the right hand side the experiment was repeated, but an unlabeled oligonucleotide with a mutated NR4A1 binding site was added into the binding reaction as a competitor for non-specific sites. A circadian binding pattern can be observed, with the highest binding at ZT8 (arrow).

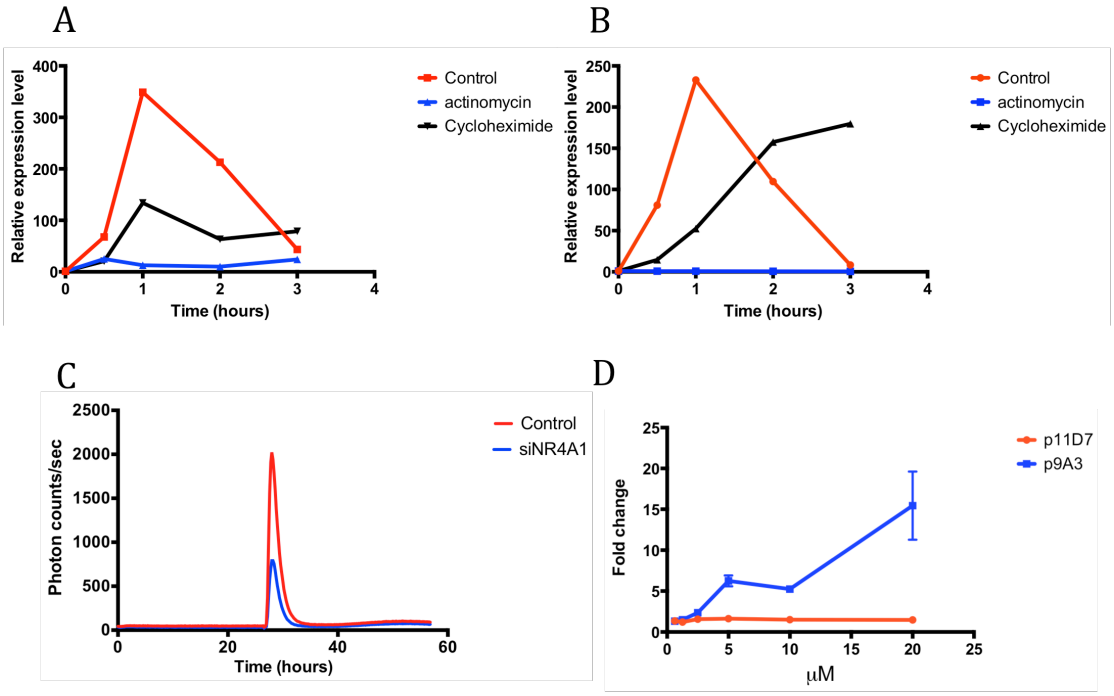


Figure 3.2 Characterization of NR4A1 as the IETF inducing lucifease expression in serum-treated HepaRG-p9A3 cells. A) The serum response, as measured by the increase of luciferase mRNA, was abolished by the treatment of the cells with actinomycin D (an inhibitor of transcription), but not by the treatment with cycloheximide (an inhibitor of protein synthesis. B) *Nr4a1* mRNA accumulation is up-regulated itself by a serum factor, (conceivably through another IETF), and the mRNA half-life appears to be longer in the presence of cycloheximide. C) siRNA-mediated knockdown of *Nr4a1* reduces the induction of the luciferase reporter. D) Luciferase expression in HepaRG-p9A3 cells could be induced by the NR4A1 agonist cytosporone B while HepaRG-p11D7 cells (negative control), expressing another serum-inducible reporter, served as a negative control to examine the specificity of this drug.

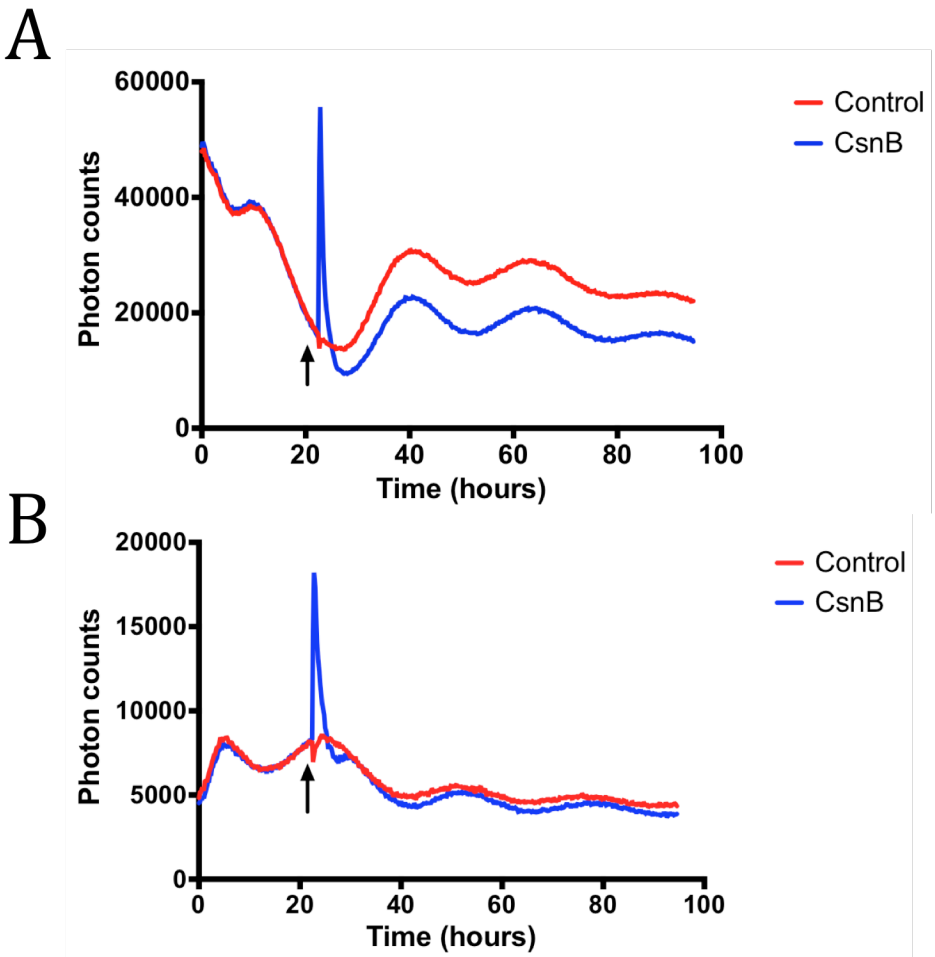
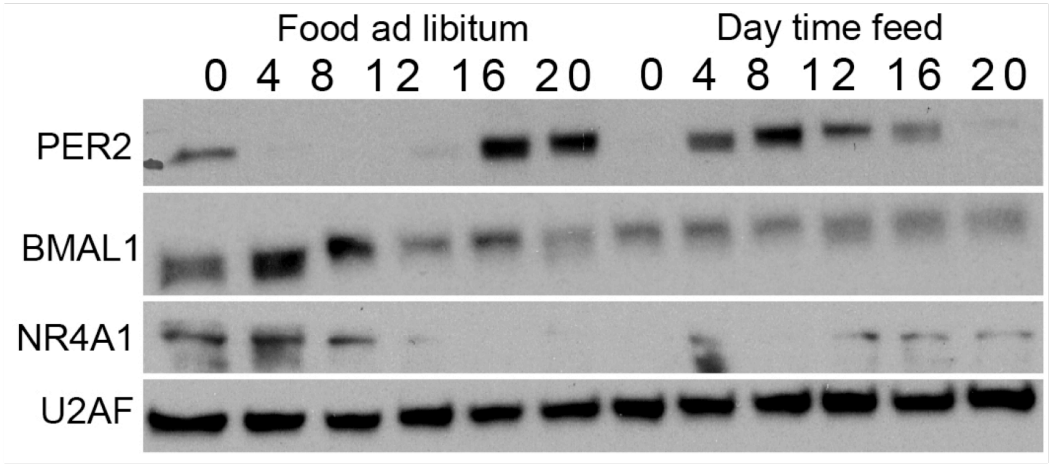


Figure 3.3 The NR4A1 agonist cyclosporone B reduces HepaRG-Bmal1luc expression. A) HepaRG-Bmal1luc reporter cells were treated with cyclosporone B(CsnB) and the bioluminescence measured continuously. B) HepaRG-Per2luc reporter cells underwent the same treatment. The magnitude of circadian Bmal1-luciferase was lower in the presence of CsnB, while CsnB had little effects, if any, on circadian Per2-luciferase expression in HepaRG-Per2luc cells. Arrow indicates the time when CsnB was added to the cells.

A



B

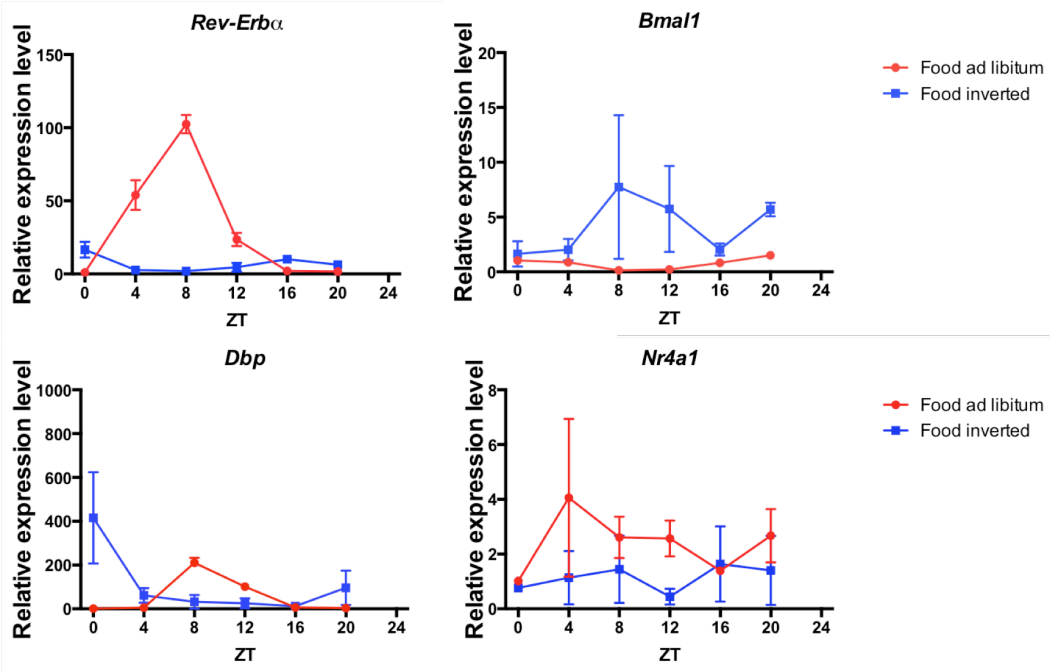


Figure 3.4 Food inversion shifts the phase of the liver circadian oscillator and NR4A1. A) Detection of the indicated proteins in a western blot experiment. The left hand side depicts the *ad libitum* condition while the right hand side depicts the situation two weeks after starting the food inversion. U2AF was used as a loading control. B) accumulation of the indicated mRNA under *ad libitum* (red) and food inversion (blue) (n = 3 for each time point).

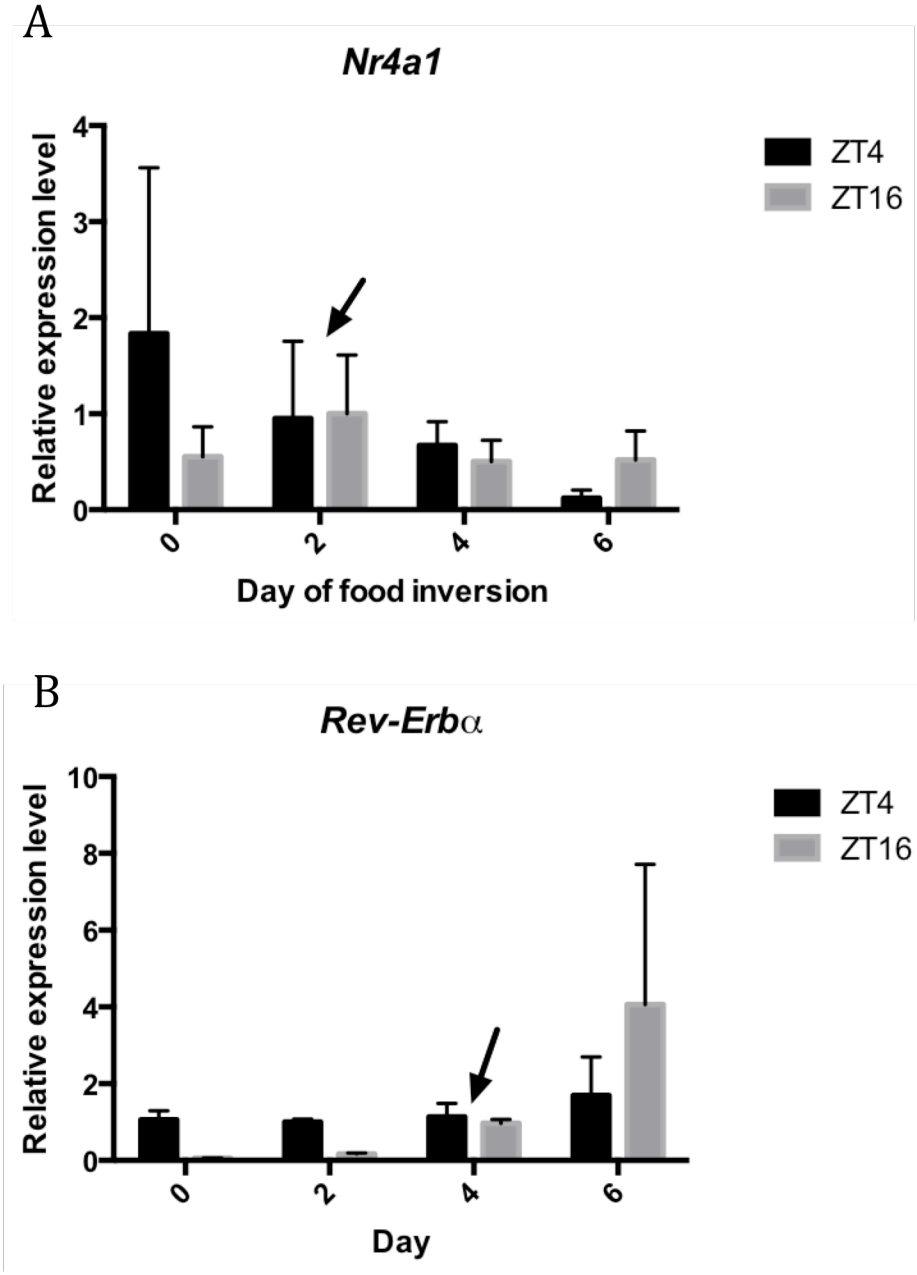


Figure 3.5 NR4A1 acts upstream of the circadian oscillator. A) Accumulation of *Nr4a1* in nuclear extracts at ZT4 and ZT16 collected 2, 4 and 6 days after the initiation of the food inversion. B) accumulation of *Nr1d1*/*Rev-Erbα*.

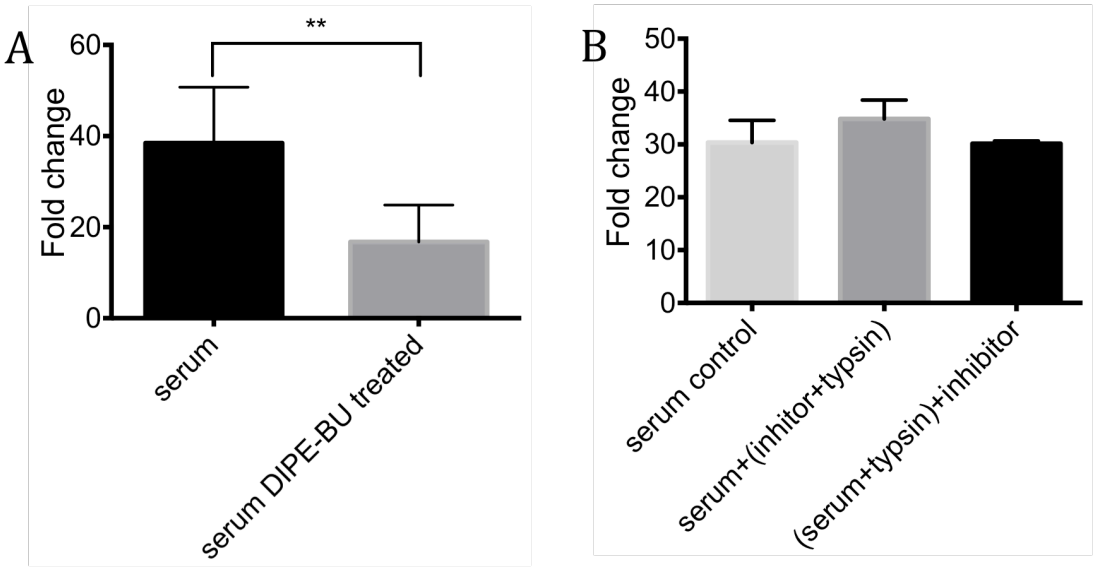


Figure 3.6 The blood-borne factors activating NR4A1 are lipids. A) Delipidation of serum by organic solvents (DIPC-BU) abolished the induction of luciferase expression in HepaRG-p9A3 cells. B) Digestion of serum proteins by trypsin does not change the induction of luciferase expression in HepaRG-p9A3 reporter. (DIPE-BU: Diisopropyl ether in butanol)

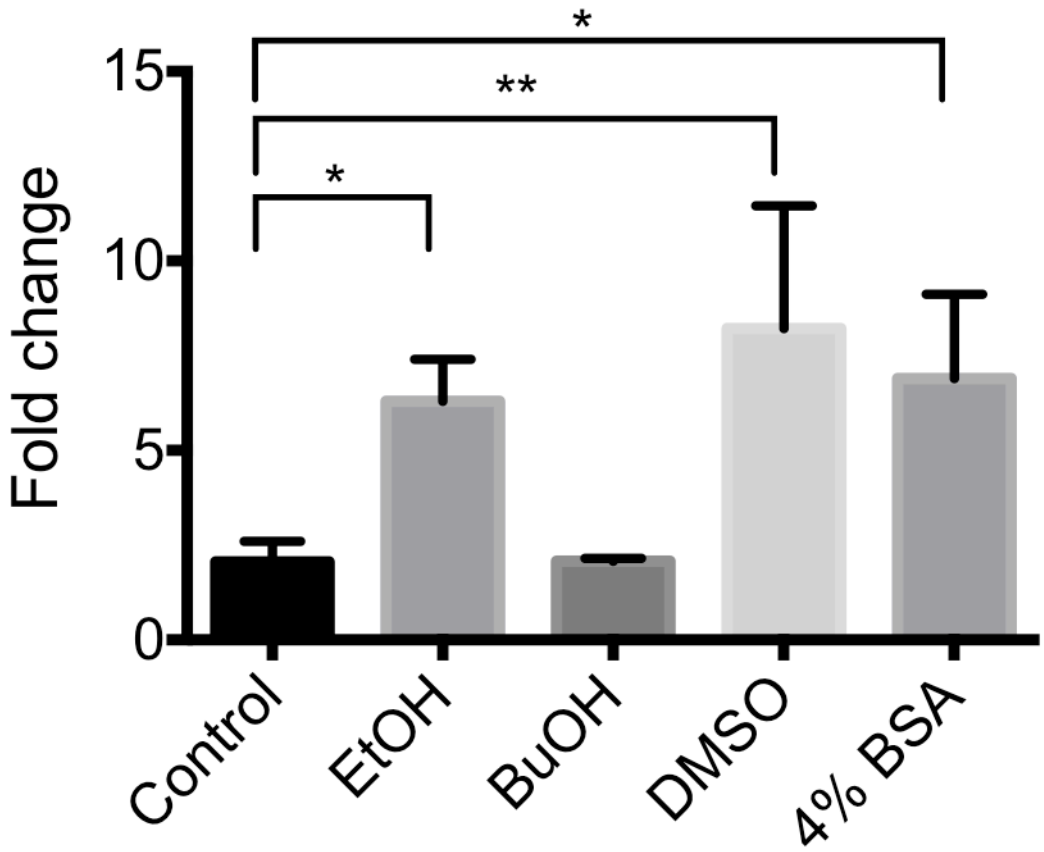


Figure 3.7 Reconstitution of lipoprotein complexes. The serum lipids were purified by organic solvent extraction. The lipids were dried and re-dissolved in different solvents before reconstitution with charcoal-treated serum proteins. Control: charcoal-treated serum without lipids. (EtOH: Ethanol, BuOH: butanol, DMSO: Dimethyl sulfoxide)

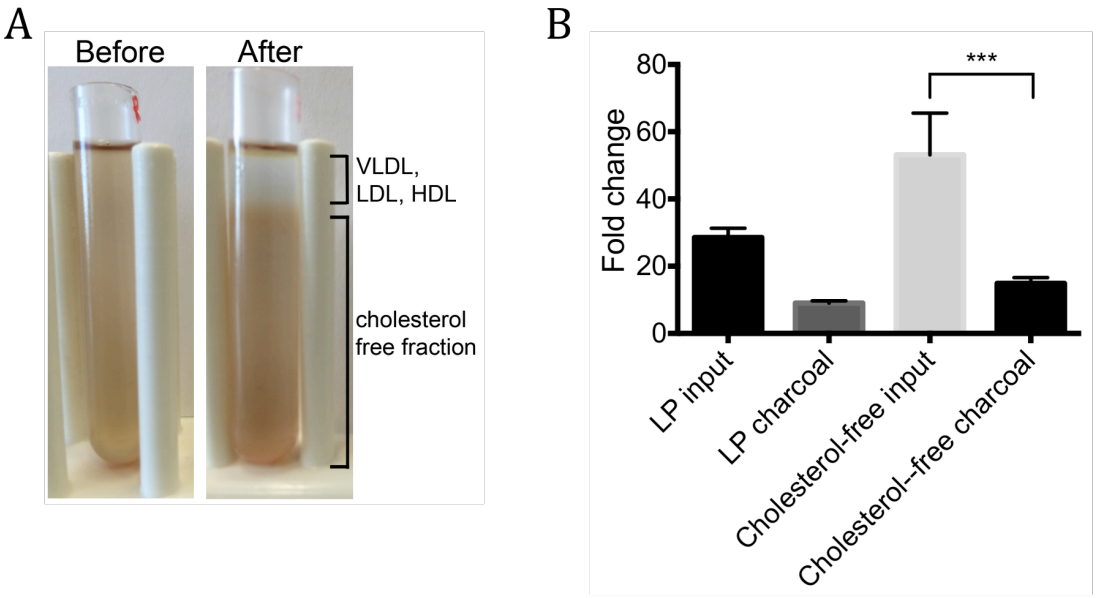


Figure 3.8 The NR4A1 inducers are present in both the cholesterol-containing lipoproteins fraction and the cholesterol-depleted fraction. A) Photograph showing the serum fractions after ultracentrifugation. B) Induction of luciferase expression in HepaRG-p9A3 cells by different fractions. Charcoal was used to remove the lipids in the serum samples. LP: Cholesterol-containing lipoproteins including VLDL (Very Low Density Lipoproteins,), LDL(Low Density Lipoproteins), HDL (High Density Lipoproteins)

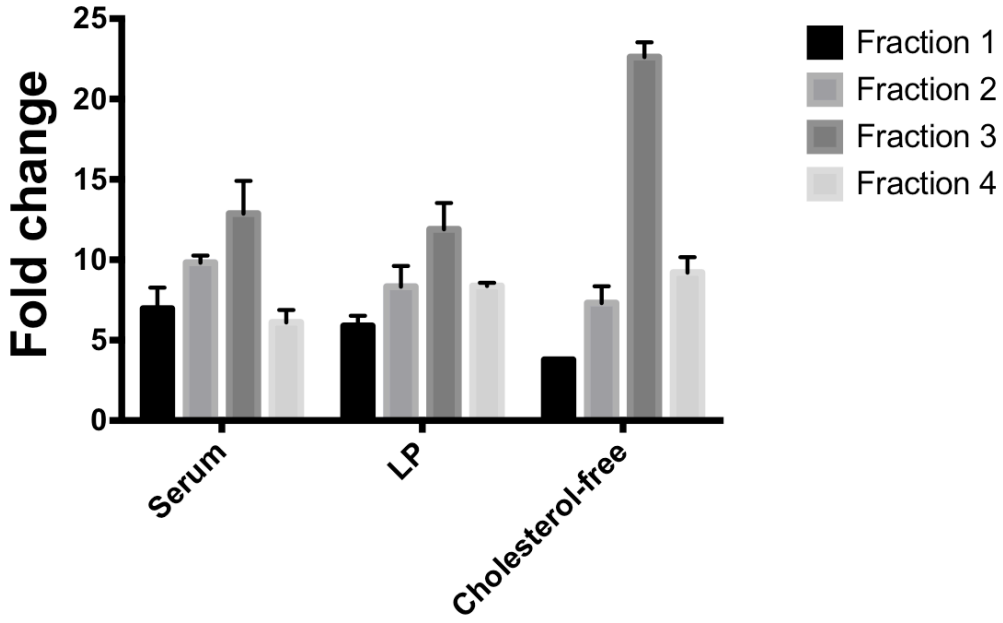


Figure 3.9 Separations of serum lipids by solid phase extraction (SPE). Lipids were extracted from serum, cholesterol-lipoproteins fraction (LP) and the cholesterol-free fractions, and then loaded onto the SPE column. Different elution fractions were collected. Fraction 1 (1% methyl acetate in hexane) contains mainly cholesterol esters, fraction 2 (2.5% methyl acetate in hexane) contains mainly triglycerides, fraction 3 (acetone) contains less well- characterized lipids, fraction 4 (methanol) contains mainly glycerophospholipids.

Supplementary information

Sequence of the synthetic promoter in the clone p9A3
5’- TAGCAAACAGATAGTCTTAATATAGCTGATGCCTCATGGAAA
GAGATAAGCGGTGTCATGGAGACC -3’

Probes used in EMSA
Probe 1
5’- GACCTGGCTCTGCAGCCTTCTAGCAAA -3’
5’- TTTGCTAGAAGGCTGCAGAGCCAGGTC -3’

Probe 2 (with NR4A1 binding site)
5’- CAGATAGTCTTAATATAGCTGATGCCTC -3’
5’- GAGGCATCAGCTATATTAAGACTATCTG -3’

Probe 3
5’- ATGGAAAGAGATAAGCGGTGTCATGGA -3’
5’- TCCATGACACCGCTTATCTCTTTCCAT -3’

Probe with mutated NR4A1 binding site
5’- CAGATAGTTGGCCGCTAGCTGATGCCTC -3’
5’- GAGGCATCAGCTAGCGGCCAACTATCTG -3’

Primers used in the RT-PCR
mouse Nr4a1
5’- ATGCCTCCCCTACCAATCTTC -3’
5’- CACCAGTTCCTGGAACTTGGA -3’

human Nr4a1
5’- CCCTGAAGTTGTTCCCCTCAC -3’
5’- GCCCTCAAGGTGTGGAGAAG -3’

human *Gapdh*:
5’- TGCACCACCAACTGCTTAGC -3’
5’- ACAGTCTTCTGGGTGGCAGTG -3’

Luciferase
5’- GCAATTCACGAATCCCAACT -3’
5’- AGGTGCTTCTCGATCTGCAT -3’

mouse *Gapdh*
5’- TCAGCTCTGGGATGACCTTG -3’
5’- TGGAAAGCTGTGGCGTGAT -3’

mouse *FoxA2*
5’- ATGCTGGGAGCCGTGAAG-3’
5’- AGGATGACATGTTCATGGAG -3’

mouse *Per2*
5’- GGCAGAGCACAACCCCTCCA -3’
5’- GCGGTGGGCTCCACATCACT -3’

mouse *Rev-Erba*
5’- GAAGTGTCTCTCCGTTGGCATGTCT -3’
5’- CGCTCTGCATCTCGGCAAGCAT -3’

mouse *Bmal1*
5’- CCAAGAAAGTATGGACACAGACAAA -3’
5’- GCATTCTTGATCCTTCCTTGGT -3’

mouse *Dbp*
5’- CGCCCACCTGGTACAGAAGGA -3’
5’- TGGGCGAGTGGGTGACCAAA -3’

4. Discussion

4.1 Discovery of new transcription factors in circadian biology

In this study, I have successfully identified and characterized two new transcription factors that have regulatory roles in the circadian clock of the mouse liver. My work demonstrated that the use of a lentivirus-based STAR-PROM screening system has advantages over the original procedure, which was based on transient transfections. The analysis of the HepaRG cells provided new candidates for the regulation of the circadian clock in the liver. FOXA2 and NR4A1 are expressed in hepatocytes but not in the osteosarcoma, U2-OS cells or fibroblasts, supporting the idea that choosing a differentiated cell line for the in vitro screening might be beneficial. Other model cell lines for cellular differentiation could be adapted to the lenti-STAR-PROM screen. For example, the 3T3-L1 cell line is a pre-adipocytes cell line, which can be readily be differentiated into mature adipocytes.

Another disadvantage of using a transient transfection system is the high and temporally variable reporter gene expression before serum induction. When studied at the single molecule level, transcription factors spend about 90% of the time searching through the genome by one-dimensional diffusion. However, the residence time increases when the transcription factor found its matching binding consensus sequence (Elf et al., 2007). The high copy number of the reporter DNA in transiently transfected cells might increase the chance of non-specific binding of the transcription factors and thus aberrant transcription initiation before the serum induction. The problem with high reporter gene expression prior to induction is that the fold change after the serum shock or the stimulation by other sources for signaling molecules would not reflect the physiological range. Moreover, a high number of STAR-PROM plasmids per cell nucleus may deplete the concentration of rare or moderately abundant transcription factors, thereby reducing the pool of active molecules. Using a lentivirus-based system solved these problems, as only a few copies of the reporter genes integrated into the genome. Therefore, the pre-induction expression is relatively low, and the fold induction of luciferase expression after the serum shock would be expected to be more potent. This is probably also the reason why we did not find any false positive clones in the secondary screen.

Third, the screen done in this study revealed a larger diversity of serum-responsive clones than the one performed with the initial STAR-PROM (Gerber et al., 2013). The screen previously done in our lab by Alan Gerber with 5% serum and U2-OS cells, yielded 49 serum-responsive clones out of about 900 clones present in the library, whereas there were only 1% of the serum-positive clones out of ~2,800 clones in my library. However, the clones discovered in my screen showed a higher diversity. The serum-responsive clones uncovered include SRF, a glucocorticoid-responsive clone, insulin- and glucose-responsive clones, cAMP-responsive clones, and two new serum-induced factors, FOXA2 and NR4A1, which affect the circadian expression of some of the circadian genes. These latter clones were not found in the previous study.

Since the beginning of 2000, owing to the development of DNA microarray and genome sequencing technologies, numerous transcriptome studies around the clock were conducted with RNA from different tissues. However, subsequent bioinformatics analysis of the promoters of the rhythmically expressed genes failed to reveal novel transcriptional regulators implicated in the control of circadian transcription. For example, Ueda and colleagues discovered REV-ERRα/ROR response elements in numerous circadian genes in expressed in the SCN and liver through such methods (Ueda et al., 2002). However, the involved transcription factors, RORs and REV-ERBs were previously discovered by our group (Preitner et al., 2002). As time went by, there were improvements in the algorithms to define rhythmically expressed genes and the search for regulatory consensus sequences in promoters or enhancers. The study by Bozek and colleagues published a more comprehensive analysis of the consensus sequences over-represented in clock-controlled genes (Bozek et al., 2009). The following table summarizes the motifs revealed by this study that are present in the 30 positive clones I found. Comparing the prediction results and the motifs from Bozek's paper, AP-1, CREB, C/EBP, SP1, AP-2 were found over-represented in clock-controlled genes in the liver. In addition, there were different nuclear receptor binding motifs that were over-represented in the predictions, including RXR, GR, LXR, VDR and T3R. Further functional tests are needed to see if all of these nuclear receptors would regulate circadian clock. In the case of GR, the role of glucocorticoid signaling in the synchronization of peripheral clocks *in vitro* and *in vivo* had been shown previously (Balsalobre et al., 2000; Le

Minh et al., 2001). RXR is an obligatory heterodimerization partner of several nuclear receptors, including retinoic acid receptor (RAR) and peroxisome proliferator activated receptor (PPAR) isoforms. Based on experiments with cultured cells it has been proposed that RAR regulates circadian gene expression in the vasculature by interacting with the core clock transcription factors CLOCK and BMAL1 (McNamara et al., 2001), but conclusive *in vivo* experiments are still outstanding. Members of the PPAR family are known to play a role in circadian lipid metabolism (Reviewed by Chen and Yang, 2014).

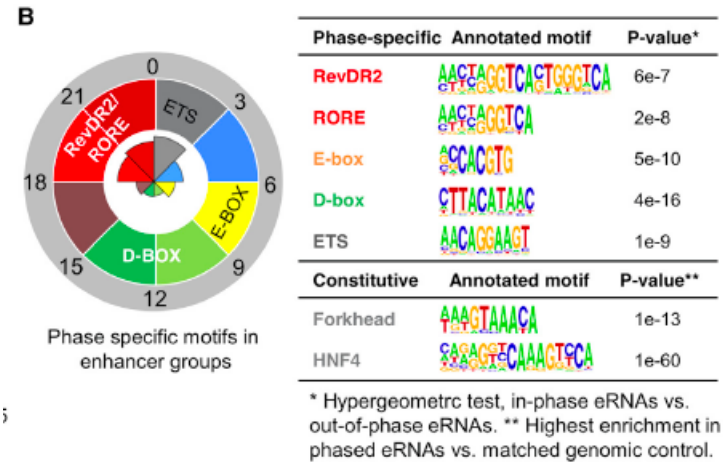
Motif	Frequency	Percentage
GATA	101	8.12
RXR	55	4.42
GR	54	4.34
AP-1	52	4.18
POU family	48	3.86
SRF	42	3.38
CREB	57	4.59
C/EBP	38	3.06
c-Jun	35	2.82
c-Fos	28	2.25
LXR	26	2.09
SAP-1	26	2.09
VDR	26	2.09
SP1	25	2.01
T3R	22	1.77
ATF	20	1.60
WT1	20	1.60
CREM	15	1.20
HNF-1	15	1.20
NF-E2	14	1.12
AP-2	14	1.12
c-Ets-1	13	1.04
c-Myb	13	1.04
Elk-1	13	1.04
NF-kappaB	13	1.04
HNF-3	12	0.96
Meis-2	12	0.96
STAT5	12	0.96

Table 4.1 The transcription factor binding motifs found in the positive clones of the STAR-PROM approach described in this thesis. In red are the motifs over-represented in clock-controlled genes according to Bozek et al., 2009.

There are other methods used to identify novel enhancers in the regulation of the circadian gene expression, including DNase I hypersensitive site mapping (Andersin et al., unpublished data), micrococcal nuclease digestion (Menet et al., 2014) and genome-wide enhancer RNA (eRNA) mapping (Fang et al., 2014). As the cis-acting regulatory sequences such as promoters and enhancers are hypersensitive to DNase I, the mapping of DNase I hypersensitive sites was widely and reliably used in the mapping of transcription factor binding sites in chromatin. This method is complementary to Chromatin Immunoprecipitation (ChIP) experiments, in which cells or tissue homogenates are treated with formaldehyde to crosslink transcription factors or other chromatin-bound proteins to DNA. The chromatins are then fragmented by sonication or nuclease treatments, and the DNA segments associated with the proteins of interest are then are immunoprecipitated antibodies with suitable antibodies. Obviously, however, ChIP experiments can only been performed for the identification of binding sites for known chromatin-associated proteins. The mapping of DNase I hypersensitive sites is a powerful strategy to find cis-acting regulatory in an unbiased manner. If the sequencing of small DNA fragments released by a mild digestion of nuclei with DNase1 is performed at high depth, it will reveal binding properties of transcription factors at the nucleotide level. Such experiments have been performed by Teemu Andersin, a previous PhD student and Postdoc in our laboratory, with liver nuclei harvested around the clock (in the framework of CycliX <http://www.cyclix.org/>, a program of the Swiss systems biology program SystemsX). The data are currently written for publication by the group of Felix Naef at the EPFL, who performed the bioinformatics analysis. They already revealed invariable information on circadian motifs, one of them being the binding site of FOXA2, one of the factors revealed by our STAR-PROM screen.

Recently discovered eRNA data was also used to find new enhancers in different contexts (Hah et al., 2013). eRNA is a new class of RNAs produced when RNA polymerase II transcribes bi-directionally at enhancer sites. Those enhancer sites are also defined by the presence of histone H3 monomethylated lysine 4 (Kim et al., 2009). Fang and colleagues used Global Run-on Sequencing (GRO-seq,) method (Core et al., 2008) to capture the nascent RNA chains synthesized to map the production of eRNA. They identified the well-characterized circadian enhancers containing binding sites like ROR/REV-ERBα binding sites, E-boxes, D-boxes and

ETS boxes (as shown in the figure 4.1). Interestingly, they also found potential forkhead and HNF4 binding sites to be constitutively functional around the clock in the liver. However, they did not specify the role of forkhead transcription factors or HNF4 in the regulation of the circadian clock in the liver. The advantage of STAR-PROM is that it provides a functional screen for the activation of the transcription factors. In our *Foxa2* conditional knockout animals, we revealed the down-regulation of *Rev-Erbα* and other core clock genes, while the phase of the circadian clock was unaffected when the animals were entrained by light. Our finding suggested that FoxA2 might be important to relay the extracellular signals to the circadian clock and to maintain robust amplitude of circadian gene expressions in the liver.



Phase-specific	Annotated motif	P-value*
RevDR2		6e-7
RORE		2e-8
E-box		5e-10
D-box		4e-16
ETS		1e-9
Constitutive	**Annotated motif**	**P-value****
Forkhead		1e-13
HNF4		1e-60

* Hypergeometrc test, in-phase eRNAs vs. out-of-phase eRNAs. ** Highest enrichment in phased eRNAs vs. matched genomic control.

Figure 4.1 Binding occupancy of response elements during the circadian cycle (adapted from Feng et al., 2014). Comparison between the presence of regulatory elements and the phase of eRNA expression in the mouse liver. The experiment suggested a time-of-day effect of transcription factors binding to their target sequence. Note the distinction into phase-specific and constitutive binding factors.

I would discuss two examples of other strategies which could also be used for the identification of new circadian regulators. Patwardhan and colleagues developed the 'massively parallel reporter assay' to study the impact of enhancer mutations on gene expression on the single-nucleotide resolutions *in vivo* (Patwardhan et al., 2012). They cloned three different mammalian enhancers with > 100,000 mutations introduced during the amplification process. Each haplotype of the enhancers were linked to the luciferase reporter tagged with barcodes. They delivered a pool of these plasmids DNA into the mouse liver through hydrodynamic tail vein injection. Liver RNA was extracted, and the barcodes counted by high-throughput RT-PCR sequencing. The promoter and the RNA expression level quantified by the tag (or the barcode) were matched by parallel sequencing. They have successfully profiled the effect of mutations in the HNF4 and HNF1 binding sites in the AldoB gene and the corresponding gene expression level. However, the major technical challenge of this technique is that the reporter transcripts were present in relatively small amounts in the total RNA. Hence, the authors suggested that enrichment methods during the RNA purification steps might be necessary to increase the sensitivity of the assay.

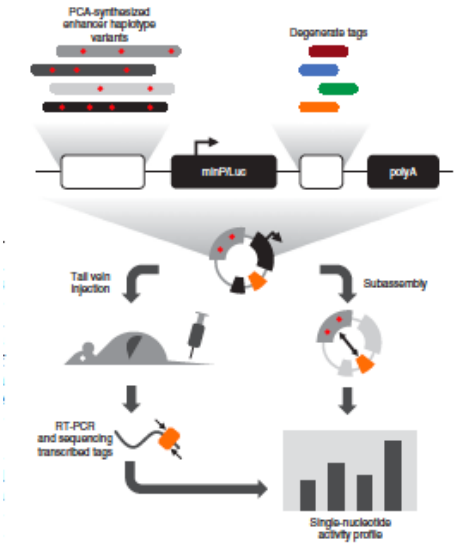


Figure 4.2 Screen to evaluate enhancer mutations in mouse liver (adapted from Patwardhan et al., 2012). The screen consisted of a library of enhancer mutations with corresponding sequence tags. The constructs were delivered to hepatocytes by hydrodynamic tail-vein injection. RT-PCR was then used to amplify the expressed tags from liver tissue, and the tags were sequenced to determine their relative expression levels.

The second study elucidated gene regulatory logic in yeast with thousands of systematically designed promoters (Sharon et al., 2012). To avoid the possibility that the sequence tag (or barcode) in the 3' of the RNA might affect reporter gene expression by mRNA stability, they put the tag at the 5' of the promoter and transformed the yeast with the synthetic promoter constructs. Rather than quantifying the reporter RNA with the tag, they separated cells with different reporter expression levels by FACS and then sequenced the promoter sequences containing the tag. In this way, they correlated the reporter expression level with the promoter sequences. As they used a programmable DNA microarray to synthesize their synthetic promoters, they could infer the regulatory logic by comparing gene expression results with custom designed variations of the promoters of interest. For examples, they assessed single nucleotide mutations, inversions of binding sites, and different copy number of the binding sites. As a result, they successfully classified the effect of single nucleotide change, inversions of the binding sites, various copy numbers of the binding sites.

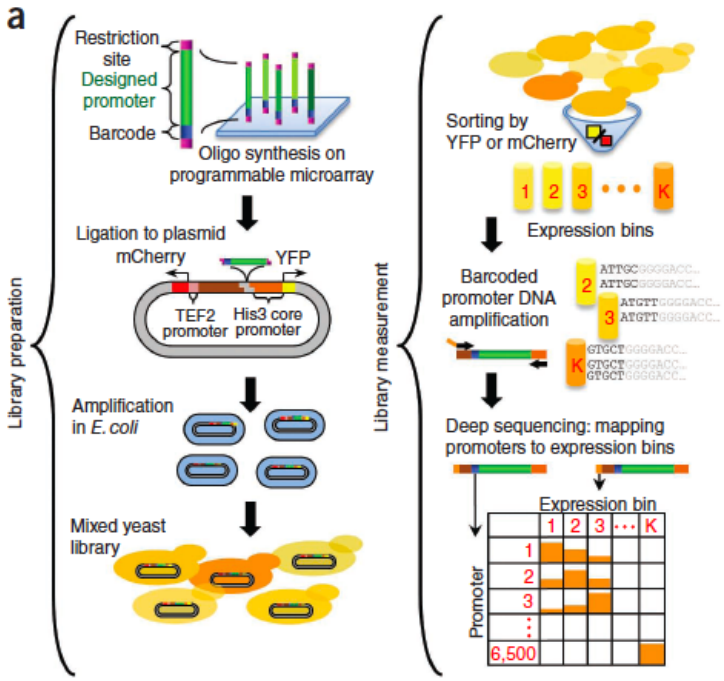


Figure 4.3 Synthetic promoter screen in yeast (adapted from Sharon et al., 2012). A DNA microarray synthesis platform was used to create a library of enhancer mutants. These were used to drive YFP expression in yeast cells and as such could be separated into different expression classes. The synthetic enhancers could then be identified by a similar bar-code strategy as described in Fig. 2

Finally, the study of the knockout phenotype of a transcription factor only gives us information on how the concerned transcription factor functions globally on lots of enhancers or binding sites in the genome. With the breakthrough of genome editing technologies like ZFNs (Zinc-finger nucleases), TALENs (transcription activator-like effector nucleases) and CRISPR/Cas9 (reviewed by Kim and Kim, 2014), individual enhancer elements can now be studied by insertion or deletion in the genome. An excellent and impressive example is the silencing of the extra chromosome 21 of cells contained from Down's syndrome patients by hijacking the X-inactivation pathway *in vitro* (Jiang et al., 2013). The researchers used ZFN to inserting the XIST (X-inactivation gene) into the chromosome 21 and successfully inactivation of one of the trisomy chromosomes 21 with the formation of structure resembled the Barr body. This gain-of-function experiment illustrated the power of genome editing tools in functional study of individual cis-regulatory elements.

4.2 Isolation of novel blood-borne signaling molecules

Discovering novel signaling pathways for the synchronization of circadian clocks in peripheral organs is one of our major goals. We started to search for blood-borne factors that induce signaling pathways in hepatocytes. Our laboratory has successfully identified SRF (Geber et al., 2013), FOXA2, and NR4A1 using STAR-PROM technology. The remaining and difficult-to-solve outstanding question is to identify the blood-borne signaling molecule(s). While proteins appear to be responsible for the activation of SRF and FOXA2, lipids are likely inducers of NR4A1. The development of more sensitive mass spectrometry and fractionation technologies rendered plasma/serum proteomics and metabolomics approaches more potent. However, most of the studies focused on clinical applications rather than on projects in basic research. Blood is routinely sampled in the clinic, therefore the composition of proteins and metabolites have become attractive resources for developing biomarkers for disease diagnosis, for example, heart failure (Dunn et al., 2011), pancreatic cancer (Bathe et al., 2011), hepatocellular carcinoma (Tsai et al., 2015) and cognitive impairment (IJsselstijn et al., 2013). The Human Proteome Organization (HUPO) launched the Human Plasma Proteome Project (HPPP) in 2002 to coordinate the efforts of 55 laboratories worldwide to identify and document human plasma proteins. In August 2005, the phase I HPPP results were published in

28 articles documenting about 3,000 plasma proteins (Omenn et al., 2005). In the on-going phase II study, they aim to cross-analyze their peptide atlas with urine, kidney and liver proteome datasets derived from other HUPO initiatives (www.hupo.org). The international effort to identify all the plasma/serum proteins would be a valuable source for the discovery of novel hormones. However, it would still be technically challenging to conduct plasma/serum proteomic studies around the clock in humans. In fact, circadian proteins must have a short half-life in the blood, and their abundance might thus be particularly low.

Blood-borne signals also attracted much attention in the aging field, given the discoveries of the "magical" rejuvenating effects of young blood in aged individuals. Young blood factors can reverse age-related impairments in cognitive functions, the synaptic plasticity in brain (Villeda et al., 2014), and the age-related hypertrophy in heart (Loffredo et al., 2013). However, the mechanisms remain elusive. The study by Loffredo and colleagues suggested that growth differentiation factor 11 (GDF11) in the young blood caused the reversal of the aging phenotype. However, recent studies by Egerman and colleagues showed that the amount of GDF11 actually increased with age and it had a deleterious effect on muscle regeneration (Egerman et al., 2015).

There were very disappointingly few new hormones discovered in recent years, and in some cases these discoveries sparked controversies. I will briefly discuss two examples of newly discovered hormones, betatrophin and irisin, and the lesson we have learned from these stories. Betatrophin (ANGPTL8) was first reported as a hormone that controls the proliferation of beta cells in the pancreas (Yi et al., 2013). Initially, the authors observed insulin resistance and beta cell proliferation in mice treated with the insulin receptor antagonist S961, a 43 amino acid long peptide. As the peptide S961 did not directly act on the beta cells to stimulate their proliferation, they looked for gene expression changes in the liver, muscle and fat tissue to find candidates for the responsible signaling factor. They found one gene, which was up-regulated in the liver when the mice were treated with S961, and named it betatrophin. Betatrophin was found to be a secreted protein with a molecular weight of about 22 kDa. After characterization, they claimed that betatrophin was indeed the factor mediating insulin resistance and beta cells expansion. However, one year after the report by Melton and coworkers appeared in press, Gusarova and colleagues

published studies on the betatrophin (ANGPTL8) knockout mice. They found that in the absence of betatrophin, the mice still developed insulin resistance and beta cell expansion under high-fat diet. In addition, their evidence supported the idea that betatrophin regulated the metabolism of triglycerides rather than glucose homeostasis (Gusarova et al, 2014; Fenzl A et al, 2014).

The exercise hormone irisin was first discovered in 2012 by Spiegelman's group. They observed the browning of fat cells when they were treated with conditioned medium of muscle cells over-expressing PGC1-α in vitro. They used bioinformatics approaches to identify transcripts that encode secretory proteins from muscle cells. FNDC5 was found to be a transmembrane protein, and the extracellular part of FNDC5 was cleaved to form the functional peptide irisin. Irisin can be detected in mouse and human plasma. They further claimed that the level of irisin could be induced by exercise, which reduced obesity and insulin resistance (Bostrom et al., 2012). Moreover, a recent study showed that irisin displayed a diurnal accumulation pattern in the blood in healthy human subjects (Anastasilakis et al., 2014). However, the subject of whether irisin is performing its purported effects - or whether it is even present at meaningful concentrations in the blood - is still controversial (Crujeiras et al., 2015). Using state-of-the-art mass spectrometry, Spiegelman and coworkers have reinvestigated the blood concentrations of irisin in human subjects. They found that the plasma concentration of irisin augments from~3.6 ng/ml in sedentary human individuals to ~4.3 ng/ml (Jedrychowski et al., 2015) in individuals performing aerobic interval exercises. While these concentrations are in the range of human protein hormones, it remains to be shown whether the 20% difference plasma concentration difference in resting and active subjects has long-lasting effects on the composition of white and beige fat or on obesity and insulin resistance.

Both studies started with a cellular process, beta cells expansion in the case of betatrophin, and browning of fat tissue for irisin. Both studies looked for secreted protein from the transcriptome of the source organ, liver in the case of betatrophin and muscle for irisin. Once they identified the candidate, they put the potential hormone to test *in vitro* or *in vivo*. As we have seen for betatrophin, however, such genetic experiments are crucial to verify the effect of a hormone *in vivo*. We also tried to

identify the source organ of the signals in blood for activating FOXA2. Intestine showed significant induction, but this could be due to the abundance of proteases present in the organ extract, which may activate PAR-related pathways.

There are several circadian lipidomics and metabolomics studies in mice and humans published recently that revealed diurnal changes in metabolites and lipids found. However, when compared across studies little or no consensus can be drawn (Ang et al., 2012; Chua et al., 2013; Dallmann et al., 2012 ; Kasukawa et al., 2012, Reviewed Gooley and Chua, 2014). Other than technical differences on normalization and bioinformatics, there are several biological aspects that need to be taken into account. The first concern is the genetic variation of the human subjects participating in the studies. The four studies compared by Gooley and Chua were conducted in four different countries, including United Kingdoms, Switzerland, America, and Japan, which suggested a very diverse genetic background from the subjects. Second, lipids and metabolites in the blood can be influenced by the composition of the food intake. Although all the studies were controlled for the calories of the food intake, the nutritional content of the food given to the subject may not be comparable in these studies. Third, the metabolisms are also affected by the gut microbes, and individuals have unique microbiome signatures. There is evidence showing that the circadian clock of the host depended on the microbial communities in the gut (Mukherji et al., 2013; Hooper et al., 2015). The intestinal microbiome displayed diurnal changes in mice and humans, which might optimize metabolism. Disturbances of this rhythmicity caused a higher risk of diseases like diabetes and obesity (Thaiss et al., 2014; Voigt et al., 2014; Leone et al., 2015). It would perhaps be more meaningful to conduct lipidomics and metabolomics studies with the aim to test the biological function of the lipids or metabolites. For example, the phosphatidylcholine 18:0/18:1 was identified as a regulator of PPARa (Liu et al., 2013). We hope that new lipidomics methods developed to study the signaling of lipoprotein complex will allow us – or the scientific community at large -to identify the activator lipid of NR4A1 in the future.

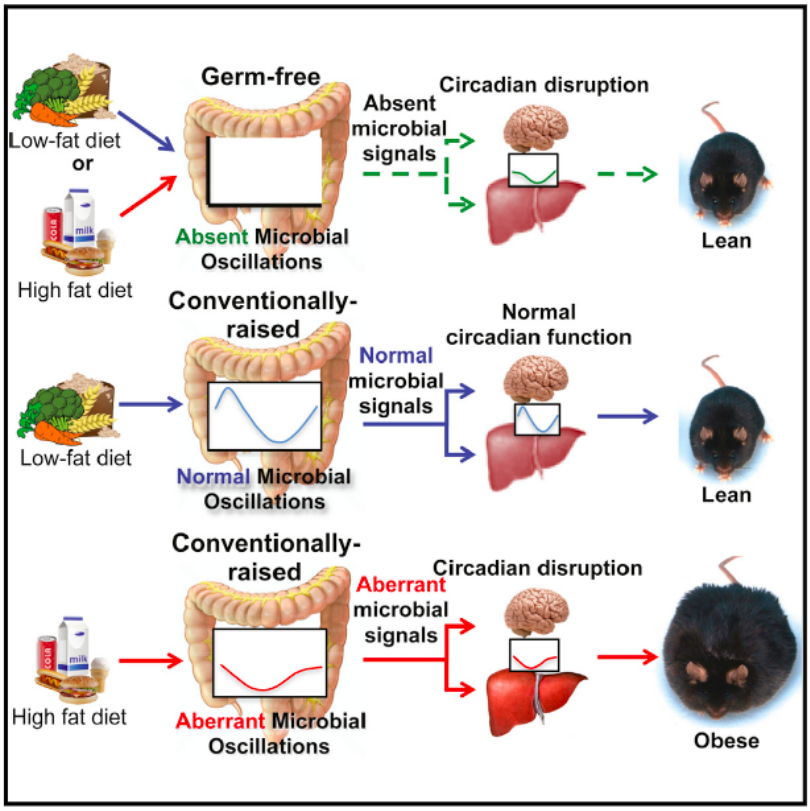


Figure 4.4 Correlation between the gut microbiota and the metabolic state of mice (adapted from Leone et al., 2015). In normal mice, there is an interaction between the gut microbiota and the circadian system of the host. Upon disruption of the circadian system, feeding with high-fat diet provokes the metabolic syndrome. Mice without microbes in their gut, however, are resistant to the adverse effects of a high-fat diet.

4.3 Final model: What is the bigger picture?

As we have seen from the organization of the circadian system in mammals, feeding rhythms can uncouple peripheral clocks from the SCN. In this project, I have discovered two independent putative pathways for the synchronization of the peripheral clocks by serum components. Conceivably, FOXA2 relays signals from the SCN to liver cells, while NR4A1 is likely to relay signals from the feeding status to liver cells. From our experimental data, FOXA2 seems to have broad and more general effects on clock genes. FOXA2 may act as a pioneer factor opening up the local chromatin and consequently act on the chromatin level rather than locally at the promoters of the clock genes. Indeed, by checking available ChIP-sequencing data I found that binding sites for FOXA1 and FOXA2 were present in nearly all of the circadian core clock genes. However, the experiments performed with the liver-

specific *FoxA2* knockout mice suggested that the circadian oscillator reacted very slowly to the loss of FOXA2. Hence, it is feasible that FOXA2 acts more like a general factor adjusting the overall activity of the circadian oscillator in the liver. A situation, in which such a general mechanism would be useful, could be during liver tissue damage, which could induce local thrombin signaling. Similarly, FOXA2 activity was shown to increase with age and to occupy more binding sites in the liver of old than in the liver of young animals (Bochkis et al., 2013). However, it remains to be seen if there are age-related changes on the circadian oscillator mediated by FOXA2.

The effect of NR4A1 may be more local than that of *FoxA2*. We have demonstrated an effect on *Bmal1*-luciferase reporter gene expression by the pharmacological activation of NR4A1. The distribution of NR4A1 binding sites in the liver is not known yet. Genome-wide ChIP study would be useful to determine the genes downstream of NR4A1. Unfortunately, *Nr4a1*-deficient mice, because of impairments in their immune system, are difficult to handle and analyze. Hence, it would be useful to generate floxed *Nr4a1* mice to obtain liver-specific knockout mice comparable to those for *FoxA2*.

There are many redundant signaling pathways that can influence circadian gene expression. Yet, peripheral clocks may have to be resilient to too many changes at the same time. As one can imagine, in real life animals are subjected to various fluctuations in environmental parameters, including those affecting the times of feeding availability and the optimal temperature range. Therefore, the effects of the systemic cues might be masked by signals directly controlled by the SCN. Only when food availability or temperature oscillations persist for long time periods would it be useful change the phase of peripheral clocks. One artificial situation in which this happens is jet lag, caused by the sudden change in time zone. Humans have not been exposed to this condition until recently, and the evolution of oscillators has thus not been influenced by such sudden changes. Nonetheless, jet lag protocols are suitable to examine phase-shifting properties of central and peripheral clocks. Our laboratory has shown that peripheral clocks of SCN-lesioned animals adapt much more rapidly to a sudden inversion of the feeding regimen. Hence the SCN, whose phase is not affected by feeding rhythms, must send out signals counteracting those emitted by feeding

rhythms (Saini et al., 2013). The signals from the SCN must be quite potent, since when the phase of the SCN is changed by large phase shifts (corresponding to that elicited by a transatlantic flight to the East Coast of the United States), the peripheral clocks adapt their phase without delay (Pascal Gos and Ueli Schibler, unpublished data).

The first layer of signals conferring timing information from the SCN pacemaker to peripheral clocks would be cues directly emanating from the SCN or SCN-controlled brain regions. For instance, after transplanting fibroblasts from a mutant mouse to a wild-type mouse, the fibroblasts became synchronized to the host organism irrespective of their defect in the circadian clock (Pando et al., 2002). The second layer would be signals elicited by food availability. These signals would be systemic signals as well, which do not necessarily reflect rhythmic SCN functions. Rather, these signals give information about the metabolic and energetic states of an organism. Nevertheless, the activity of this second information layer would still be influenced by the SCN, simply via controlling rest-activity rhythms and thus the fasting-feeding time. However, under restricted feeding conditions imposed in the laboratory it becomes apparent that food is a dominant *Zeitgeber* for the liver circadian clock. The signals involved in this second layer are also of medical relevance. It has been shown that restriction of subjects to ‘normal’ feeding times with the smartphone app could help increase weight loss and improve sleep quality (Gill and Panda, 2015).

Finally, in the third layer information is exchanged between cells of the same organ or tissue. For example, liver oscillators maintain phase coherence even in arrhythmic SCN-lesioned animals. Gap-junction, and/or paracrine or humoral factors may facilitate such an information exchange within an organ, but further experiments are necessary to validate or invalidate this conjecture. However, when cells, such as Rat1 or NIH 3T3 fibroblasts, are kept in culture, they have to be synchronized by external cues such as a serum shock or temperature cycles. Hence, the organ-specific coupling is lost under these conditions..

Taken together, many signaling pathways can affect peripheral circadian oscillators, probably even at the same time. Why does this situation not create

confusions within the system? Transcriptional mechanisms involved in the control of gene expression can act either by activation or repression. Because of the complexity of the mammalian genomes each gene has a specific array of transcriptional regulatory sites or enhancers. These enhancers integrate external information in relayed by activating or repressing transcription factors. Only the cooperative binding of multiple transcriptional regulators allows for gene expression above the transcriptional noise. In addition, due to the coupling of the cells within an organ, an inappropriate activation of 1% or even 10% of the total cells would probably not be harmful to the organism. Clearly, further experiments are necessary to understand how a given organ can integrate the whole plethora of blood-borne, neuronal, metabolic, and temperature-dependent signals.

4.4 Conclusions

A technical advance in STAR-PROM screening allowed for the analysis of blood-borne factors affecting immediate early transcription factor activation in hepatocytes. This work also served as an independent verification of the usefulness of the STAR-PROM screen to identify such factors. Among the identified clones there were glucocorticoid-, insulin-, and glucose-responsive clones, in addition to several clones with binding sites for SRF, FOXA2 and NR4A1. A variety of signaling cascades activated the FOXA2 reporter cell line, including those depending on Ca^{2+}, the MEK1/2 kinases, the protease-activated receptor 1 (PAR-1), and tyrosine kinases. NR4A1 was activated by a yet to be identified hydrophobic ligands. Both FOXA2 and NR4A1 affected different aspects of liver circadian oscillators. Many further experiments are necessary to pinpoint the precise functions of these two transcription factors in the circadian timing system *in vivo*.

5. Appendix

News and views article “Grab the wiggly tail: new insights into the dynamics of circadian clocks” coauthored with Dr. Jürgen A. Ripperger. This is a commentary on the original research article “Cryptochrome 1 regulates the circadian clock through dynamic interactions with the BMAL1 C terminus” by Xu et al. Both articles published in the journal Nature structural and molecular biology in June 2015.

Grab the wiggly tail: new insights into the dynamics of circadian clocks

Ka Yi Hui & Jürgen A Ripperger

How do molecular interactions determine the period length of a circadian oscillator? In mammals, a disordered region of the BMAL1 transcription factor that is able to interact with activators or repressors seems to perform this function.

Circadian clocks are commonly found in bacteria, fungi, plants and animals[1]. They are used to synchronize periodical environmental cues, such as the light-dark cycle, to internal regulatory programs in an organism. The basic components and the genetic circuitry of circadian clocks are quite well characterized. However, there is only limited information on circadian dynamics at the structural level, especially in higher organisms. In this issue, Partch, Liu and colleagues[2] report that the intrinsically disordered C-terminal region of the transcriptional activator BMAL1 alternately discriminates between interaction with p300 (CBP) and with CRY1 to set the pace of the mammalian circadian clock.

The canonical molecular mechanism underlying circadian rhythms consists of both transcriptional and post-translational feedback loops[1] (**Fig. 1**). In principle, alternating gene expression driven by activators and repressors generates 24-hour rhythms. Activators drive the expression of repressor proteins and other circadian clock–controlled (CCC) genes. The repressor proteins accumulate to a sufficient threshold concentration that enables them to bind to the activator complex and repress gene expression. Upon subsequent degradation of the repressor proteins, the activators are reactivated, and a new cycle of gene expression occurs. In *Drosophila*, the activators are Cycle and Clock, which interact with the repressors Period (Per) and Timeless[3]. A similar molecular architecture is found in the mammalian system. However, gene duplication has increased the number of players: BMAL1 and BMAL2 are the mammalian homologs of *Drosophila* Cycle[4], and PER1 and PER2 are the homologs of Period[5]. The Cryptochrome (CRY) proteins, originally functioning as blue-light sensors in plants and *Drosophila*[6], became part of the repressor complex in the mammalian circadian system[5]. The organization of the functional domains of these proteins is shown in **Figure 1**.

To understand the fundamental question of how the transcriptional and post-translational feedback loops work, the relevant interactions between activators and repressors must be determined in detail. The crystal structures of the BMAL1–CLOCK heterodimer and the CRY1–PER2 complex have recently been solved[7,8], but no structure of the proposed functional BMAL1–CLOCK–CRY–PER quaternary complex (**Fig. 1**) is currently available. Nevertheless, there are biochemical data that suggest how the repressor complex binds to the BMAL1–CLOCK complex to inactivate transcription. Ye *et al.*[9] previously generated quadruple-knockout cell lines deficient in *Cry1*, *Cry2*, *Per1* and *Per2*, and used them to show that a CRY1–BMAL1–CLOCK ternary complex directly represses CCC genes such as *Nr1d1* and *Dbp*, whereas PER–CRY complexes act instead by detaching the BMAL1–CLOCK complex from DNA.

However, the above model does not sufficiently explain the wide spectrum of period-length phenotypes observed when individual clock genes are knocked out in cells. For example, $Cry1^{-/-}$ cells have a short period length, whereas $Cry2^{-/-}$ cells have a long period length[10]. Previously, Kim and Forger proposed an alternative 'stoichiometric balance' model[11], in which the repressors bind directly to the activators rather than to DNA. Their model

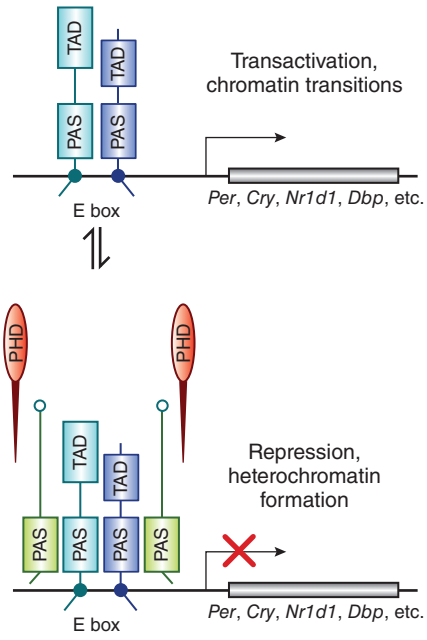


Figure 1 Model of the mammalian circadian oscillator. The activity of the BMAL1 (dark blue) and CLOCK (light blue) heterodimeric activator is rhythmically counterbalanced by binding of PER (green) and CRY (red oval) repressor complexes to alternate between states of transcriptional activation (top) and repression (bottom) at the promoters of circadian target genes (several example circadian genes shown). Corresponding chromatin transitions support both active and inactive states. Solid circles represent basic helix-loop-helix domains, which mediate binding to the E-box motif (5′-CACGTG-3′). The PAS domains and C termini of CRY mediate protein-protein interactions. TAD, transactivation domain; PHD, PHD domain.

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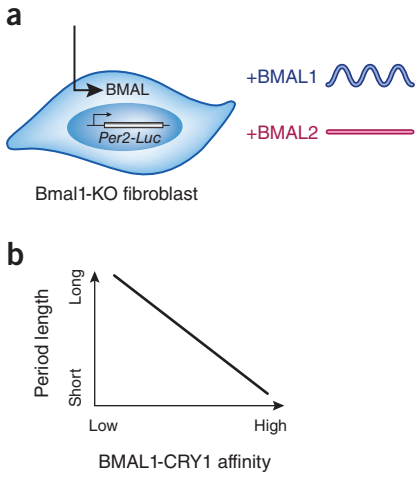

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Figure 2 Analysis of the differences between BMAL1 and BMAL2. (**a**) Complementation assay to reconstitute the circadian rhythmicity of *Bmal1*-knockout fibroblasts with different BMAL proteins. BMAL1 (blue) can restore rhythmic *Per2*-luciferase activity (*Per2-Luc* reporter), but BMAL2 (pink) cannot; this allowed domain-swap and mutation experiments to identify the functionally relevant BMAL1 C terminus. (**b**) Simplified correlation between the period length measured in the complementation assay and the affinity of the CRY1-BMAL1 interaction.

predicts that the optimal ratio between such activators and repressors is around 1:1, and any coarse violation of this ratio would lead to less robust circadian rhythms. Interestingly, *in vivo* it is primarily the concentration of the repressing factors that cycles with circadian amplitude[12], and this could potentially fine-tune the competition between both types of factors. However, the model grouped clock components into only activators or repressors. Because the mammalian *Cry1, Cry2, Per1* and *Per2* paralogs of the clock genes have important roles in generating robust circadian rhythms, measurements of the binding affinities of these clock components with BMAL1 and BMAL2 are necessary to obtain a more comprehensive picture of the functional interactions that underlie their activities.

In this issue, Patch, Liu and colleagues[2] examined the role of the *Bmal1* (official symbol *Arntl*) paralog *Bmal2* (official symbol *Arntl2*) in circadian-clock function (**Fig. 2a**). They found that BMAL2 could not compensate for the function of BMAL1 in *Bmal1*-knockout cells. A series of domain-swapping experiments between BMAL1 and BMAL2 revealed that the C-terminal region of BMAL1 was essential in generating circadian rhythms. They further showed that CRY1 repressor and p300 (CBP) coactivator compete for binding to the same C-terminal domain of BMAL1. Biochemical and biophysical assays enabled them to identify specific mutations in the C-terminal region of BMAL1 that correlate the period length of the complemented fibroblasts with the binding affinity of CRY1 to BMAL1 (**Fig. 2b**). Their findings agree with the observed phenotype of Cry-deficient cells: $Cry1^{-/-}$ cells would mimic a low-affinity state with short circadian period length, and $Cry2^{-/-}$ cells, because of extended accumulation of CRY1 (ref. 13), would mimic a higher-affinity state that gives rise to a longer period length.

From these observations, Partch, Liu and colleagues[2] have provided compelling evidence for a competition mechanism that sets the pace of the circadian oscillator. Interestingly, this mechanism is located in the intrinsically disordered C-terminal region of the BMAL1 protein. Disordered structures are involved in protein-protein interactions and are known to mediate important transcriptional regulatory pathways such as that of p53 (ref. 14). Their inherent flexibility may provide some advantages over ordered structures (**Fig. 3a**) because a disordered structure vastly increases the number of potential interaction surfaces and permits multiple different proteins to associate with a single underlying peptide sequence. Moreover, a disordered structure is thermodynamically favored because it has higher entropy than an ordered structure; hence, creating order by interaction with another protein is actually disfavored, and this may impart further specificity to the interaction.

Coimmunoprecipitation experiments show that BMAL2 and BMAL1 have similar binding affinities for CRY1, despite the observation that BMAL2 cannot rescue BMAL1 deficiency. This most probably reflects differences in the behavior of their C termini (**Fig. 3b**). One simple scenario predicts that post-translational modifications such as phosphorylation would govern their different possible interactions. Alternatively, dynamic interactions of the C terminus with CRY or p300 (CBP) may generate rhythmic transcription. The interaction of CRY and p300 (CBP) with BMAL2 may not be as flexible as that with BMAL1 for generating rhythmic gene expression. Further experiments are necessary to address these possibilities.

In summary, a competition mechanism based on the dynamic interactions provided by a disordered structure is a key component that determines the period length of the mammalian circadian oscillator. These observations represent a further step toward understanding the regulatory network underlying the mammalian oscillator, by focusing on a structural detail and molecular flexibility. Many more dynamic interactions may be involved in

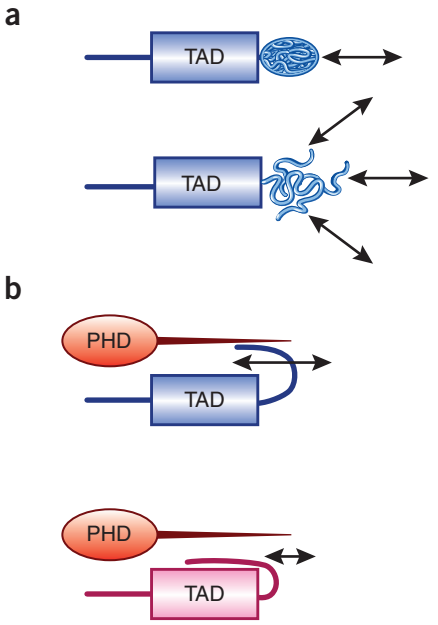

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Figure 3 Properties of disordered proteins may explain differences in the function of BMAL1 and BMAL2. (**a**) Intrinsically disordered proteins (bottom) have higher entropy and an increased number of potential interaction surfaces than ordered polypeptides (top). (**b**) BMAL1 (dark blue) and BMAL2 (pink) have roughly the same affinity for CRY1 (red ovals), but only the disordered C terminus of BMAL1 may be dynamic enough to permit efficient competition of activators and repressors.

the fine-tuning of the clock mechanism, and a combination of genetic, biochemical and biophysical experiments could be uniquely suited to resolving this issue. In addition, similar detailed structural and functional studies of related clock proteins that extend the findings reported here would help to address the fundamental question of how the regulatory complexity of the mammalian circadian clock arose during evolution.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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