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Mechanism for 12 Hr Rhythm Generation by the Circadian Clock

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SUMMARY

In addition to the well-known circadian rhythms in animal behavior, cell biology, and gene expression, there are also 12 hr rhythms. The mechanism giving rise to these 12 hr rhythms is not clear. We worked with the hypothesis that observed 12 hr rhythms in gene expression are the results of an interplay between components of the circadian clock. Analysis of mouse liver expression data indeed revealed a strong circadian component in observed 12 hr gene expression rhythms. We show theoretically that pairs of circadian transcription factors with certain circadian phase relationships can give rise to these 12 hr rhythms, if binding noncompetitively to the promoters of regulated genes. We took an inventory of circadian transcription factors in mouse liver using available microarray data and matched these to predicted binding sites in the promoters of genes displaying 12 hr rhythms. Binding sites for transcription factor pairs with phase relationships predicted by theory were overrepresented in these promoters.

INTRODUCTION

Up to 10% of all mammalian genes, depending on tissue, measurement, and analysis method, are regulated by the circadian clock and thus oscillate in their expression with a period of around 24 hr (Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002; Hughes et al., 2009; Keller et al., 2009). In addition to these circadian genes, a smaller group of genes (on the order of 1% of all genes) with a 12 hr expression rhythm was recently discovered in mouse liver (Hughes et al., 2009). In subsequent studies, similar 12 hr rhythms were found in other genome-wide mouse liver studies (Vollmers et al., 2009; Hughes et al., 2012), and a 12 hr gene expression rhythm of *Ire1α* with corresponding protein concentration oscillations was studied in depth (Cretenet et al., 2010). This rhythm was proposed to be related to the response to endoplasmatic reticulum (ER) stress, an accumulation of misfolded proteins in the ER that typically reaches a maximum twice a day. These maxima correspond to the peak of insulin-induced processes and the glucagon-induced processes, respectively, which normally are separated by around 12 hr and are associated with ER stress (Cretenet et al., 2010). Others have reported 12 hr rhythms of NAD^+ levels in mouse liver (Ramsey et al., 2009), of VIP2 receptor in rat SCN neurons (Cagampang et al., 1998), of the expression of core clock genes *mPer1* and *mPer2* in mouse bone marrow (Chen et al., 2000), of several core clock genes in mouse spleen and testis (Yamamoto et al., 2004; Liu et al., 2007), and of enzymes involved in lipid metabolism in mice fed a high-fat diet (Kohsaka et al., 2007).

The molecular origin of cellular circadian rhythms is well studied and partially understood. A small network of transcriptional feedback loops has been shown to generate 24 hr rhythms (Sato et al., 2006; Zhang and Kay, 2010; Hogenesch and Ueda, 2011). This network involves core clock genes, feeding back on E/E′ box, D box, and ROR regulatory elements in their own promoters, as well as of output circadian genes. However, the molecular basis of the 12 hr rhythms is unknown. Noting that the circadian genes in liver exhibit a continuous phase distribution (i.e., distribution of peak gene expression), Hughes et al. (2009) proposed that an interplay between two different circadian regulatory components (e.g., transcription factors [TFs]) with opposite phases might induce a 12 hr rhythm of a given gene. Support for this theory was provided by a subsequent study by Hughes et al. (2012). Here, the authors studied mice with a disrupted *Clock* gene resulting in loss of circadian rhythms. Interestingly, the 12 hr rhythms in the livers of these mice were also lost. However, conditionally rescuing circadian rhythms in the SCN of those animals resulted in the appearance of 24 hr rhythms of many liver genes including many genes that exhibited 12 hr rhythms in wild-type animals. This points to 24 hr signals from the SCN being one of two (or more) components that interact to produce 12 hr rhythms. However, how such an interplay may be implemented within cells has not been thoroughly investigated.

One gene regulatory design that has been studied by circadian biologists is that of two or more TFs competing for the same binding site on a promoter. Although, as will be shown in the present study, this process cannot be expected to generate 12 hr rhythms, it can in fact be exploited to boost the amplitude of circadian gene expression. This was shown by Mitsui et al. (2001) for circadian TFs binding to promoter D box elements, and it was formulated quantitatively by Ueda et al. (2005) as a phenomenological model for the expression of a gene governed by a promoter element that binds two competing circadian TFs. In subsequent work, constructs of a reporter gene driven by different combinations of circadian regulatory elements (D boxes, E/E′ boxes, ROR elements) were used to investigate combinatorial circadian gene regulation (Ukai-Tadenuma et al., 2011). In this case, the TFs do not compete for the same

site; rather, they bind to separate regulatory elements that both are able to control gene expression. In general, competitive and noncompetitive regulation should be expected to obey different kinetic laws (Cornish-Bowden, 2004). Ukai-Tadenuma et al. (2011) reported the resulting circadian expression patterns as being mostly consistent with contributions of the different regulatory elements being a superposition of sine waves. This was formulated as a so-called phase vector model. However, some striking anomalies were observed when the constructs used in that investigation were driven by circadian TFs with large phase differences, including the emergence of 12 hr rhythms.

A phenomenon similar to the 12 hr rhythms is the circatidal rhythms in behavior and physiology of certain marine animals that live in tidal zones (Wilcockson and Zhang, 2008). These rhythms persist in a constant environment, like circadian rhythms, but with a period of slightly more than 12 hr, corresponding to one-half of the lunar day (24.8 hr). Two main hypotheses about the nature of the circatidal rhythms have been put forward. One hypothesis assumes two separate circalunidian (with a period of 24.8 hr) oscillators running in antiphase and superposing to generate this rhythm. The other hypothesis posits that the circadian clock through an unknown mechanism is able to generate the circatidal rhythm as well, possibly in conjunction with a dedicated circatidal clock (Wilcockson and Zhang, 2008).

In this paper, we focus on the potential of the core circadian clock as the origin of 12 hr rhythms of some cellular components. The basic premise is that in a given cell type, there is a core circadian clock that gets entrained by external circadian cues that may have their origin in a central pacemaker (the SCN in mammals), or in, e.g., activity and feeding patterns. In turn, TFs that are part of the core circadian clock generate circadian rhythms in clock-controlled genes (CCGs). In addition, the external circadian cues may directly generate 24 hr rhythms in an additional group of genes (Vollmers et al., 2009). These genes together with the core clock and the CCGs comprise the set of all circadian genes (Figure 1A). We focus here on separating the mechanisms that would allow the circadian genes and possibly the external circadian cues to interact to produce 12 hr rhythms, from those that would not. In turn, 12 hr gene expression rhythms generated in this way may be the cause of 12 hr rhythms in an additional layer of genes. We state a simple general gene regulatory principle for the generation of dominant second harmonic outputs by a core oscillator and outline plausible cellular implementations of this principle, whereby 12 hr rhythms can be generated by the circadian clock. Finally, we report a waveform analysis of 12 hr rhythms in gene expression in the liver of mice held in constant darkness (Hughes et al., 2009), as well as a bioinformatic analysis of the promoters of these 12 hr genes, and present empirical evidence for the proposed mechanism.

RESULTS

To set the stage for the analysis, we briefly define two basic gene regulatory motifs that one may encounter in circadian biology. First, we have the circadian OR funnel (Figure 1B, upper). This is a competitive circadian *cis*-regulatory motif, where two or more circadian TFs compete for the same binding site, as proposed by Ukai-Tadenuma et al. (2011). In this case, amplitude may be boosted or quelled, depending on the phases of the involved TFs. Conceptually, this is the circadian analog of what has been coined the OR funnel activity motif by Chechik et al. (2008). Second, we have the circadian AND funnel (Figure 1B, lower). This is a noncompetitive circadian *cis*-regulatory motif, where two or more circadian TFs regulate the same gene, binding at least two different distinct binding sites. Conceptually, this is the circadian analog of the AND funnel activity motif of Chechik et al. (2008).

In the following, we derive the properties of circadian and 12 hr rhythms that may arise from these circadian activity motifs. We will then proceed to analyze gene expression data and promoter sequences in order to connect the theoretical insights to empirical evidence.

Competing Circadian TFs Produce 24 Hr Rhythms

We first consider previous ideas of how different circadian TFs interact at promoters to produce specific circadian outputs and show that the origin of 12 hr rhythms cannot be explained by these. Phenomenology must ultimately be connected to underlying physics of protein-DNA binding and gene expression. The phase vector model of Ukai-Tadenuma et al. (2011) is purely phenomenological. It successfully describes a substantial part of observed circadian gene expression patterns but cannot account for the production of higher harmonics. This prompted us to investigate the biophysical roots of the phase vector model, and more generally, gene expression regulated by different circadian TFs. Bintu et al. (2005) have taken an inventory of different modes of gene regulation based on equilibrium statistical mechanics as outlined in the seminal paper by Shea and Ackers (1985). We start with the circadian OR funnel. In the simplest instance, two TFs compete for the same TF binding site, as illustrated in Figure 1B, upper scheme. Bintu et al. (2005) obtained an equation that computes the normalized (v = 1 without TFs) gene expression rate v as a function of the concentrations of the two TFs:

$$v \approx \frac{1 + \gamma_1 x_1 + \gamma_2 x_2}{1 + x_1 + x_2}, \qquad \text{(Equation 1)}$$

where x_1 and x_2 denote normalized concentrations of TF1 and TF2, respectively (see Extended Results for more precise definitions). Here, the operational meanings of the γs are fold activation $(\gamma > 1)$ or fold repression ($\gamma < 1$, where, for instance, $\gamma = 0.1$ would mean 10-fold repression). The numerator and denominator of Equation 1 correspond to sums of the TF concentrations, as in the phase vector model. Moreover, Ueda et al. (2005) demonstrated that if TF1 is a circadian activator and TF2 is a circadian repressor appearing 12 hr out of phase (antiphase), the resulting gene expression follows a high-amplitude circadian rhythm.

We show by a relatively simple argument (see Extended Results) that the circadian OR funnel always generates only circadian rhythms, as long as Equation 1 is considered to hold. We here let two simple scenarios illustrate this general principle. When considering each of these scenarios, it might be tempting to expect 12 hr rhythm generation, but in fact, only circadian rhythms are possible. As a first scenario, assume that TF1 and TF2 are both circadian proteins, whose circadian oscillations can be well described by sine waves. Assume further that the

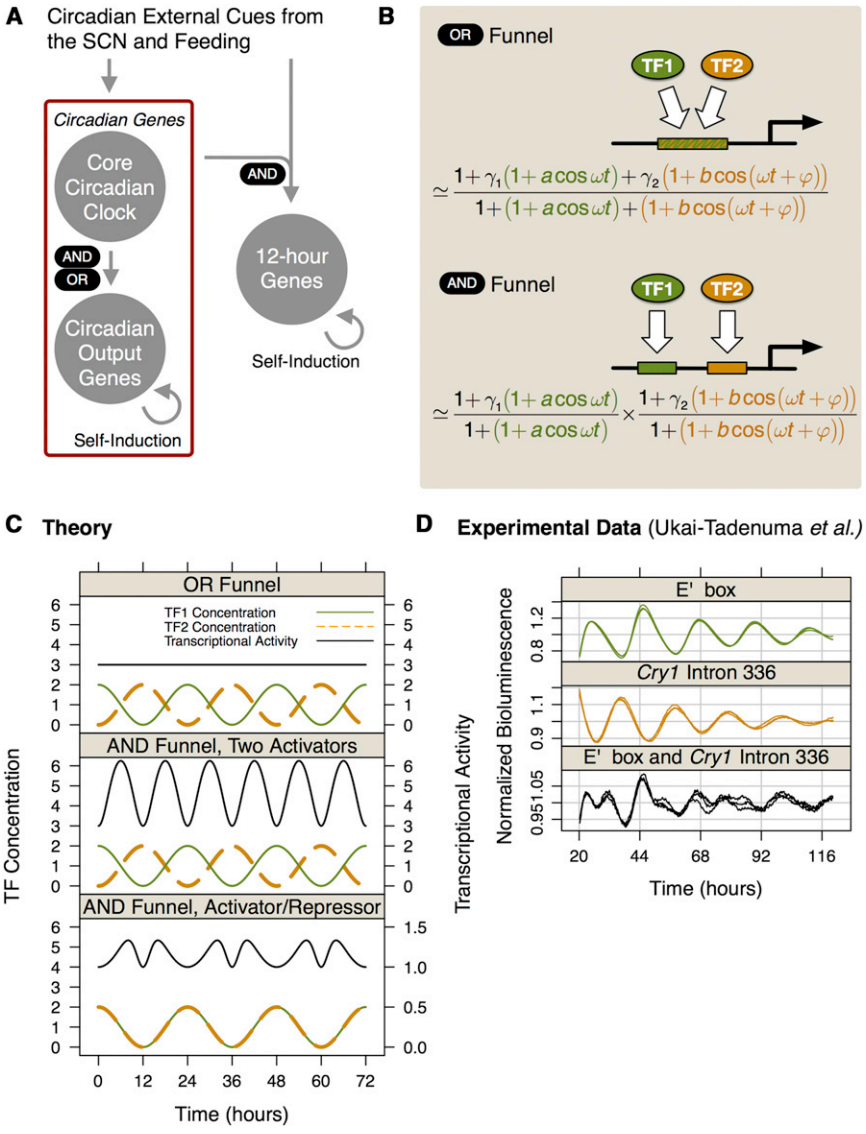


Figure 1. Effect of Two Circadian TFs on Gene Expression

(A) Circadian cues, the circadian TF cascades, and circadian activity motifs combine to a bird's eye view of circadian and 12 hr rhythm generation. We posit that the core circadian clock generates circadian genes in CCGs using both AND and OR funnels. Because some CCGs are themselves TFs, some CCGs are secondary CCGs controlled by these TFs. This is denoted "Self-Induction" in the figure. Core clock TFs or CCG TFs can generate 12 hr rhythms using AND funnels. Some 12 hr genes are themselves TFs (see main text) and may thus control secondary 12 hr genes (self-induction). External circadian signals can also be responsible for 12 hr rhythms using AND funnels, and 12 hr external cues may also exist.

(B) Upper view shows a circadian OR funnel (a circadian activity motif; see Discussion). In this motif, two circadian TFs (green and orange) compete for the same TF binding site on a promoter. This is the case, e.g., for the circadian TFs DBP, TEF, HLF, and NFIL3 (E4BP4), which all bind to D box promoter elements. In the case of two activators (e.g., DBP and TEF), the normalized activity of the TFs can be approximated as $1+a\cos\omega t$ and $1+b\cos(\omega t+\varphi)$, respectively, where φ is a phase shift separating the acrophases of the two TFs, and a and b are the relative oscillation amplitudes of the respective TFs. As derived in the analysis by Bintu et al. (2005), the transcriptional activity can then be expected to proportionally follow Equation 1, resulting in the formula given in the figure (the contributions from the respective TFs are highlighted in the corresponding color). Lower view shows a circadian AND funnel. In this motif, two circadian TFs bind to separate sites on the promoter. Acting independently of each other, the concerted gene activity should be proportional to the product of the saturating binding probabilities of the TFs, as given in Equation 2, which results in the formula given at the bottom of the figure.

(C) Upper view is an example of the dynamics of an OR funnel. If the phase shift is approximately half a period (solid green and orange curves), the oscillations cancel each other, and the gene expression rate is constant (black line). Parameters are frequency $\omega = 2\pi/24\ \mathrm{hr}^{-1}$, phase shift $\varphi = \pi$, relative amplitudes $a = b = 1$, and fold-change factor $\gamma_1=\gamma_2=4$. Middle and lower views are examples of 12 hr rhythms produced by circadian AND funnels. As shown in the main text, two circadian activators (or repressors) appearing in antiphase could give rise to 12 hr rhythms, as could an activator and a repressor that both appear at the same phase. Parameters are frequency $\omega = 2\pi/24\ \mathrm{hr}^{-1}$, relative amplitudes $a = b = 1$, phase shift $\varphi = \pi$, corresponding to 12 hr (middle panel) and $\varphi = 0$ (lower panel), respectively. The fold-change factors are $\gamma_1 = \gamma_2 = 4$ (middle panel) and $\gamma_1 = 4$, $\gamma_2 = 0$ (lower panel), respectively.

(D) Experimental proof of principle of 12 hr rhythms generated by a circadian AND funnel, as found by Ukai-Tadenuma et al. (2011). A bioluminescence reporter plasmid was fused to an SV40 promoter with different regulatory elements. Upper panel shows E′ boxes that were fused to the reporter, and circadian rhythms peaking at CT 3.5 were observed. Middle panel illustrates an intron of the *Cry1* gene containing ROR elements (binding different circadian TFs compared to the E′ box) that was instead put in front of the promoter, and circadian rhythms peaking at CT 17.6 were observed. Lower panel shows both E′ boxes and the *Cry1* intron that were fused to the promoter. Because these are distinct elements binding different sets of circadian TFs, this is an example of a circadian AND funnel. In line with the theory presented here, the result was a time course with a clearly visible 12 hr component. Raw bioluminescence data from the original experiments were kindly provided by M. Ukai-Tadenuma and H. Ueda, then normalized by a least-square fitted fifth degree polynomial. A 20-hr-long transient was omitted. For each experiment, n = 3.

See also Figure S1.

oscillations in TF1 and TF2 are similar but antiphase (specifically for this illustration, let $x_1 = 1 + a\cos\omega t$; $x_2 = 1 + a\cos(\omega t + \pi)$, where $\omega = 2\pi/24\ \mathrm{hr}^{-1}$, and a is the normalized oscillation amplitude). The resulting concerted influence of the two TFs will not be a 12 hr rhythm: rather, by insertion into Equation 1, we obtain a constant gene expression rate $v = (1 + \gamma_1 + \gamma_2)/3$, i.e., the antiphase activators cancel out. This is a consequence of the simple fact that $\cos\omega t + \cos(\omega t + \pi) = 0$. This corresponds to two vectors pointing in opposite directions in the phase vector model of Ukai-Tadenuma et al. (2011). Thus, two competing activators

appearing roughly in antiphase are predicted to produce only low-amplitude circadian oscillations. This first scenario is illustrated in Figure 1C (upper panel). Another scenario that might be suggestive of 12 hr rhythm generation is that of one circadian activator (TF1) and one circadian repressor (TF2) appearing simultaneously (in-phase), i.e., $x_1 = x_2 = 1 + a\cos\omega t$, but $\gamma_2 = 0$, to model full repression. Inserting into Equation 1, one observes that the numerator and denominator will be cosine oscillations with exactly the same phase. In the phase vector model, this corresponds to two phase vectors pointing in the same direction. Also in this case, the result is a purely circadian oscillation in which the repressor damps the overall gene expression amplitude. In all instances, circadian OR funnels, where circadian TFs compete for the same regulatory element, are unlikely to produce significant higher harmonics such as overt 12 hr rhythms (see Extended Results for a detailed analysis).

Circadian TFs Binding to Separate Binding Sites on the Same Promoter Can Produce 12 Hr Rhythms with Alternating Peak Heights

Many circadian genes contain different, nonoverlapping binding sites for circadian TFs (Yamamoto et al., 2004; Ueda et al., 2005). This situation should in general be expected to give rise to different kinetics compared to the case of competing TFs discussed above, and accordingly, Bintu et al. (2005) derived a distinct general formula for gene expression as a function of noncompeting TF concentrations. We asked what functional effect two circadian TFs each binding to its own distinct site on a promoter could have on the resulting gene expression. Such a design represents the circadian AND funnel (Figure 1B, lower). For this case, the normalized gene expression rate may be approximated as (Bintu et al., 2005)

$$v \approx \frac{1+\gamma_1 x_1}{1+x_1} \times \frac{1+\gamma_2 x_2}{1+x_2}, \qquad \text{(Equation 2)}$$

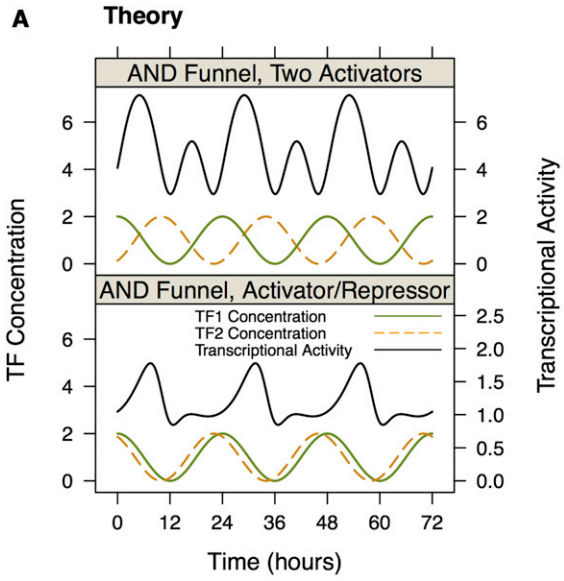
with notations as described above. This formula can produce a pure 12 hr rhythm out of purely 24 hr rhythms. To see this, assume that TF1 and TF2 are circadian activator TFs, i.e., $x_1 = 1 + a\cos\omega t$, and $x_2 = 1 + b\cos(\omega t + \varphi)$, where a and b are relative oscillation amplitudes. TF2 is phase shifted by φ with respect to TF1. We let the phase mismatch φ correspond to phase advances between 0 and 12 hr, i.e., $0 \le \varphi < \pi$. Fourier expanding each factor of Equation 2 to its first harmonic gives $v \approx C(1 + f_1\cos\omega t) \times (1 + f_2\cos(\omega t + \varphi))$, where C is a constant, and f_1 and f_2 are the amplitudes of the oscillations in gene activation caused by the activators. Carrying the multiplication out and collecting terms, we arrive at this approximation for the normalized gene expression:

$$v \approx C(A_0 + \underbrace{A_1\cos(\omega t + \psi_1)}_{\text{Phase Vector Model}} + \underbrace{A_2\cos(2\omega t + \psi_2)}_{\text{Second Harmonic}}), \qquad \text{(Equation 3)}$$

which then evidently can be decomposed into a constant term $A_0 = 1 + (f_1 f_2\cos\varphi)/2$, a circadian term with amplitude $A_1 = ((f_1)^2 + (f_2)^2 + 2f_1 f_2\cos\varphi)^{1/2}$ and a phase ψ_1 given by Equation S4, and into a second harmonic term with amplitude $A_2 = f_1 f_2/2$ and phase and $\psi_2 = \varphi$. The circadian term precisely corresponds to the phase vector model proposed by Ukai-Tadenuma et al. (2011), which explains the success of that model in describing many experimental data. However, the second term is a second harmonic representing 12 hr rhythms, which is missing in the phase vector model. The first harmonic, A_1, decreases to a minimum when the phase mismatch between the two activators is 12 hr (i.e., $\varphi = \pi$). If additionally the oscillation amplitudes are equal (i.e., $f_1 = f_2$), then the circadian term vanishes ($A_1 = 0$), and the gene expression rate follows a pure 12 hr rhythm. This is illustrated in Figure 1C (middle panel), where two antiphase circadian TFs with equal amplitude (i.e., $x_1 = 1 + a\cos\omega t$; $x2 = 1 + a\cos(\omega t + \pi)$) are plotted along with the resulting gene expression rate according to Equation 2. The resulting gene expression pattern follows a clear 12 hr rhythm. The same argument can be used to suggest two further scenarios for 12 hr rhythm generation. First, if the first TF is an activator and the second a repressor, the resulting gene expression pattern can follow a 12 hr rhythm if the two TFs have the same phase. This is because expression is low both at the peak of the repressor (maximal repression) as 12 hr earlier and later at the trough of the activator (minimal activation). Only at the in-between points there will be high gene expression, and hence, the period will be 12 hr. This is illustrated in Figure 1C (lower panel). Second, if both circadian TFs are repressors, the resulting gene expression can again follow a 12 hr rhythm if the TFs are antiphase, which is a mirror case of two activating TFs. In conclusion, the concept of two circadian TFs binding to distinct, nonoverlapping promoter elements to produce circadian AND funnels is an attractive explanation for the observed 12 hr rhythms in gene expression observed by Hughes et al. (2009).

A synthetic circadian AND funnel was constructed by Ukai-Tadenuma et al. (2011). In that study, different constructs of TF binding sites were fused to an SV40 promoter coupled to a bioluminescence reporter and were introduced in NIH 3T3 cells. Subsequently, time courses were recorded, and phases were measured. E′ boxes, promoter elements that bind the circadian CLOCK and BMAL1 (Arntl) TFs, among other basic-helix-loop-helix TFs (see also below), produced strong circadian rhythms peaking at circadian time (CT) 3.5 (Figure 1D, upper panel). When instead an intron of the *Cry1* gene was fused to the promoter, circadian rhythms peaking at CT 17.6 were recorded (Figure 1D, middle panel). This intron contains functional binding sites for the ROR family of nuclear receptors. In a further experiment, E′ boxes and the intronic sequence with ROR binding sites were both fused to the promoter. Given the circadian activity patterns of these TF binding elements, and given that they bind distinct sets of TFs, this constitutes a clean synthetic circadian AND funnel. In accordance with the theory above, the bioluminescence time course produced by this construct has a clearly visible 12 hr component (Figure 1D, lower panel). This experiment constitutes a proof-of-principle validation of the theory presented above.

In a living cell, the phase differences of the TFs in a circadian AND funnel will never be exactly 12 or 0 hr. Therefore, we analyzed how deviations from 12 (or 0 hr in case of activator-repressor combinations) affect the waveform. We found that



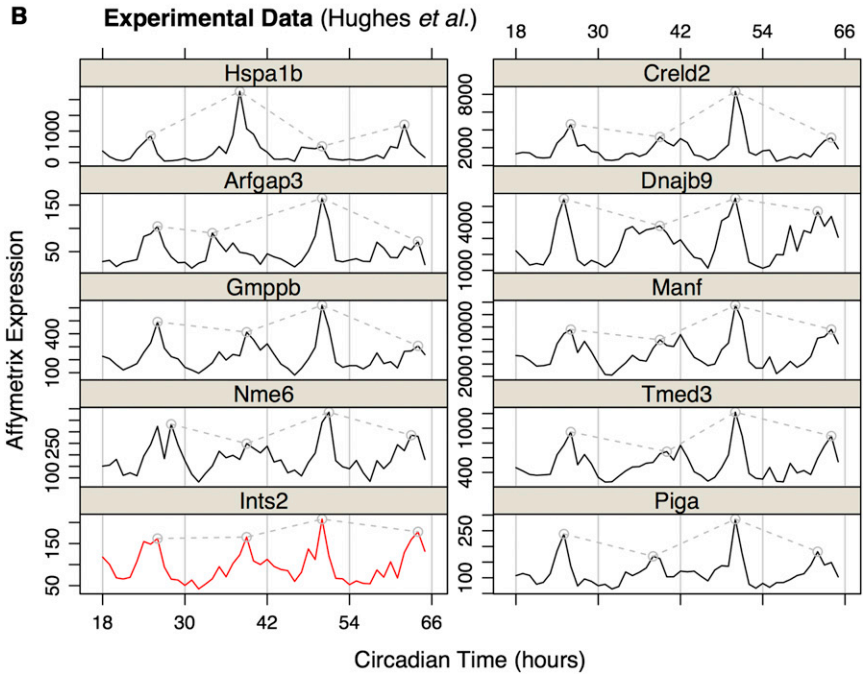


Figure 2. 12 Hr Rhythms Have Alternating Peak Heights

(A) A circadian AND funnel with two activators 10 hr out of phase results in a 12 hr rhythm with alternating peak heights (upper panel). The same is true for an AND funnel with an activator and a repressor out of phase by 2 hr.

(B) The pattern of alternating peak heights is highly overrepresented among the 197 time courses of 12 hr genes. Out of the 197 genes, 110 exhibit alternating peak heights (compared to 49 expected by pure chance; $p < 2.2 \times 10^{-16}$, binomial test). Shown are the ten 12 hr genes with the largest relative oscillation amplitudes. Only one of these time courses (red) does not match the predicted alternating peak height pattern. The alternating peak height pattern is defined by alternating positive and negative slopes of straight lines between the peaks (gray dashed lines).

small deviations of up to 2 hr from the optimal phase difference still result in visible 12 hr rhythms. The terms A_1 and A_2 in Equation 3 tell us precisely how the amplitudes of the different harmonics vary with the phase difference between the TFs. An example of this is shown in Figure S1, where there is a phase difference interval of around 4 hr in which the 12 hr component dominates. Importantly, however, these rhythms have alternating peak heights. An illustration of this phenomenon is given in Figure 2A. This is a general feature of 12 hr rhythm generation by circadian AND funnels, as explained and proven in mathematical detail in the Extended Results. In the same way, we were able to show that unequal relative amplitudes of the two TFs in a circadian AND funnel lead to 12 hr rhythms where the troughs have alternating depths (see Extended Results for proofs).

Also noteworthy is that if one TF in an AND funnel has a circadian rhythm and the other TF has a 12 hr rhythm, the resulting waveform has an 8 hr component in addition to circadian and 12 hr components (see Extended Results for the derivation of this result). Interestingly, 8 hr rhythms were indeed observed by Hughes et al. (2009), although in quite few genes.

The synthetic proof of principle shows that the circadian AND funnel can produce 12 hr rhythms, in accordance with theory. However, an analysis of genome-wide data is necessary to assess whether this mechanism could be common. Specifically, if our theory that many 12 hr rhythms arise through circadian AND funnels were correct, then a pattern of alternating peak heights should be detectable and common in experimental data. Furthermore, the promoters of genes with 12 hr rhythms should be enriched for binding sites for at least two circadian, antiphase activators (or repressors), and/or combinations of one activator and one repressor with the same phase. Both these predictions were investigated next.

12 Hr Genes Have Alternating Peak Heights

Because of biological variability, circadian TFs that form circadian AND funnels cannot be expected to be exactly 12 hr out of phase (for two activators or two repressors) or exactly in phase (for activator-repressor combinations). Therefore, our theory leads us to expect that observed 12 hr rhythms should exhibit alternating peak heights. To examine if this is indeed the case,

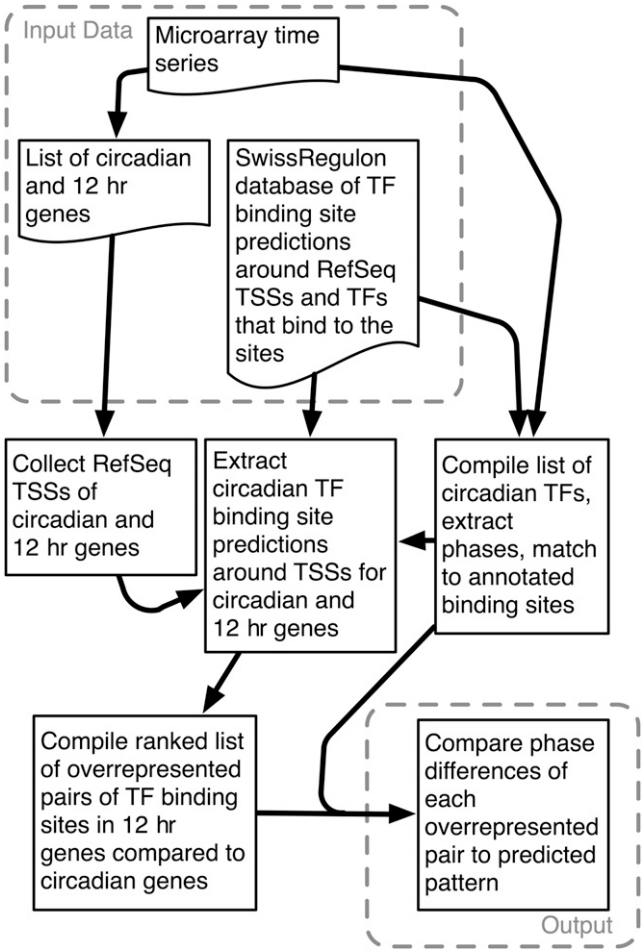


Figure 3. Workflow of the Bioinformatic Analysis

Our input data consist, first, of lists of circadian and 12 hr genes, respectively, based upon 48 hr microarray-based time series of gene expression with a sampling interval of 1 hr (Hughes et al., 2009). The second data source is the SwissRegulon database, which consists of predicted TF binding sites for 331 different TFs to 19,622 mouse gene TSSs, as annotated in the RefSeq database. The workflow briefly describes how these data were integrated in order to analyze phase differences of circadian TFs that bind to 12 hr genes.

we analyzed data from Hughes et al. (2009), a gold standard genome-wide CT series microarray data set, based on mouse liver samples, spanning 48 hr with a sampling rate of one per hour. From these data, we collected 197 time courses corresponding to genes with transcripts for which 12 hr rhythms were robustly detected in the original study (Experimental Procedures). At the troughs, the signal-to-noise ratio deteriorates, but patterns in the peak heights should be readily detectable. Thus, we screened for alternating peak heights in the time courses, as predicted by our theory. Assuming statistically independent peak heights, the probability for such a pattern to arise by chance is 25%, corresponding to 49 of the 197 time courses. However, we found that out of the 197 time courses, 110 (56%) exhibited alternating peak heights. This number vastly exceeds that expected by pure chance ($p < 2.2 \times 10^{-16}$, binomial test). Out of the ten time courses with the highest amplitudes, nine exhibited this alternating pattern, as shown in Figure 2B. This strongly suggests that the circadian clock generates the 12 hr rhythms because alternating peak heights imply underlying circadian components. Furthermore, this is consistent with a model where slightly misaligned circadian AND funnels are responsible for a large part of the observed 12 hr rhythms in gene expression, although other mechanisms cannot be ruled out at this stage.

An Inventory of Circadian TFs

As described above, 12 hr gene expression rhythms may be generated by pairs of circadian TFs binding to separate binding sites on the same promoter. For clean 12 hr rhythm generation, these TFs of a pair should appear roughly in antiphase if both are acting as activators or if both are repressors. For pairs consisting of an activating and a repressing TF, the two TFs should appear at similar phases. By contrast, circadian TFs competing for the same or overlapping binding sites would be expected to always generate 24 hr rhythms. These considerations prompted us to investigate the promoters of the 12 hr genes in order to find pairs of TF binding sites overrepresented in these promoters, compared to the promoters of regular circadian genes. Furthermore, we wanted to investigate the phases of the TFs binding to these binding site pairs and compare these to the predicted phase relationships for 12 hr rhythm generation. In order to do so, we first took an inventory of circadian TFs, their phases, and the DNA sequence motifs that they bind to. From the data set of Hughes et al. (2009), we extracted time series of 2,728 circadian genes (Figure 3; Experimental Procedures), for each of which we calculated a circadian phase given in CT. This circadian gene list was matched against a list of 340 well-defined TFs, which comes with annotations of target DNA sequence motifs (the SwissRegulon database described by Pachkov et al., 2007; see Experimental Procedures). As a result, we obtained a list of 53 circadian TFs—16% of all annotated TFs (see Table S1 for the complete list).

This represents a significant expansion of the core circadian clock output pathways as they are currently understood. The 53 circadian TFs turned out to map to 38 DNA sequence motifs, hereafter referred to as circadian motifs (CMs). Hence, some circadian TFs compete for the same promoter binding site.

Circadian TFs Competing for the Same Binding Sites Coordinate Their Phases

We found seven CMs with more than one circadian TF competing for binding to it. These CMs represent circadian OR funnels (Figure 1B). The first three were the familiar E box, D box, and ROR elements, to which it is already known that several circadian TFs bind with phases that maximize the amplitude (Figure 4). In addition, our analysis revealed MITF as a putative circadian TF that binds to E boxes in liver. Interestingly, MITF is expressed at a phase close to that of the other E box binding TFs.

Four additional regulatory elements with competing circadian TFs were discovered: the SMAD, RFX, E2F, and RXR elements (Figure 4). Interestingly, the expression of the corresponding circadian TFs in these new cases is in phase. There are good reasons to expect the phase coherence of the actual TFs to follow

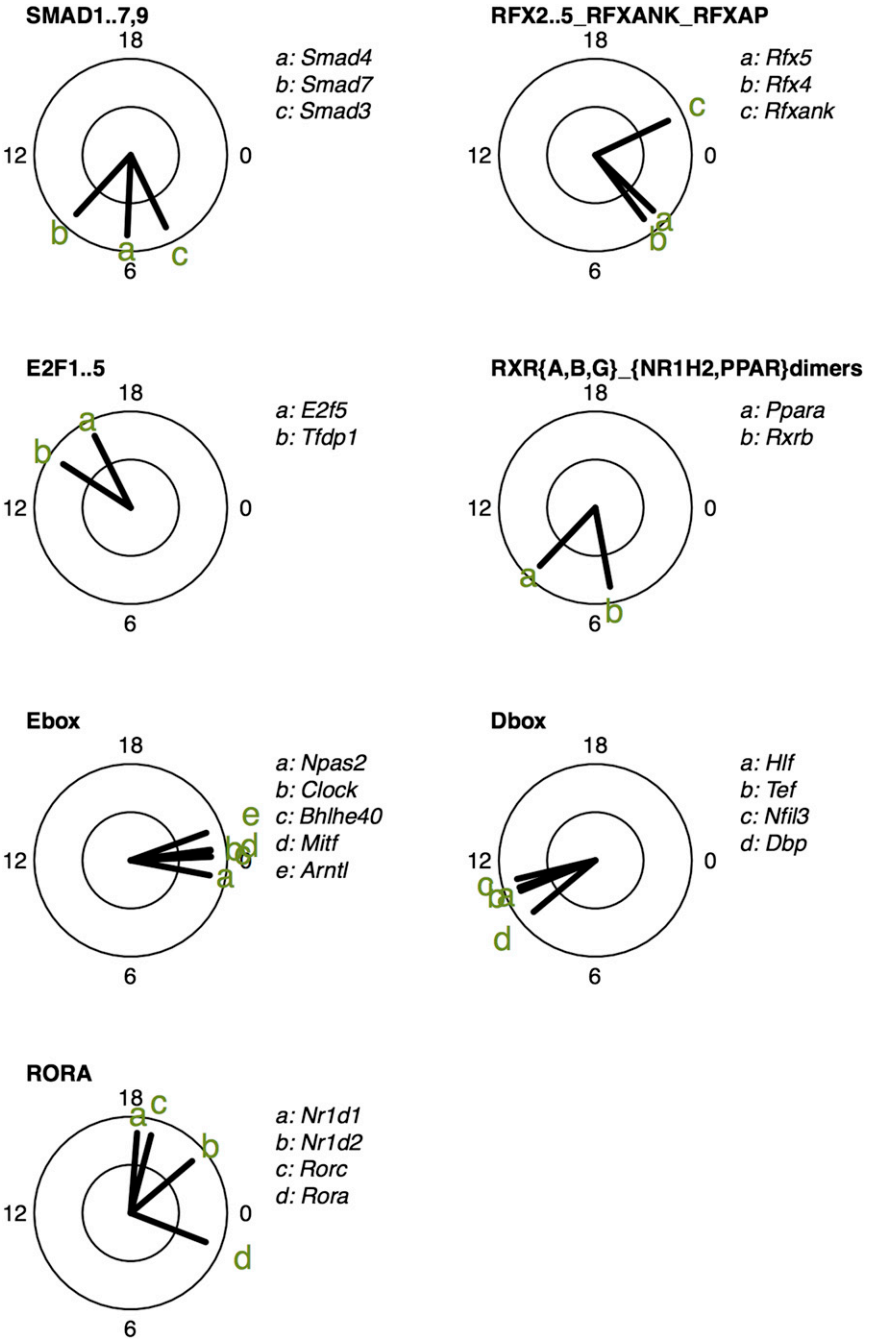


Figure 4. DNA Sequence Motifs Bound by More Than One Circadian TF

We define CMs as DNA binding motifs (from SwissRegulon) to which one or more circadian TFs bind. Here, the circadian phases of the mRNA concentration (Affymetrix microarray data) of TFs (italics) that compete for the same binding sites (SwissRegulon DNA sequence motifs, in bold text) on promoters are visualized. For the repressors NFIL3 (E4BP4) and BHLHE40 (DEC1), the phases were inverted by 12 hr to reflect the maximal transcriptional activity. The three familiar circadian CMs (E box, D box, and ROR element) were found, as well as four other putative CMs. The phases of the involved TFs of each of these seven CMs are apparently coordinated. See also Table S1.

that of their transcripts (see Extended Results). Thus, the regulation of these elements appears coordinated to maximize oscillation amplitude of the resulting circadian gene regulation. However, the regulation at least by the SMADs and by E2F and DP1 is complicated (see Table S1) and requires further investigation before firmer conclusions can be drawn. In any case, this analysis strongly supports a model where circadian TFs competing for the same regulatory element have their phases coordinated in order to maximize the output amplitude.

Overrepresented Pairs of Circadian TF Binding Sites in Promoters of 12 Hr Genes Follow the Predicted Phase Pattern

Based on the analysis so far, we hypothesized that antiphase circadian-activating (or -repressing) TFs binding the same promoter as an AND funnel (Figure 1B) could be inducers of 12 hr gene expression rhythms. Likewise, in-phase AND funnel combinations of circadian activators and repressors should be able to produce the same effect. In search of evidence for this, we examined predicted TF binding sites in promoters of genes with 12 hr and circadian gene expression rhythms, respectively (the 197 12 hr genes could be unambiguously mapped to 166 unique promoters; see Experimental Procedures). Our goal here was to examine pairs of circadian TF binding sites—circadian AND funnels—overrepresented in the promoters of 12 hr genes compared to circadian genes, to find out if these pairs exhibit phase relationships as predicted above. Although single predictions of TF binding sites must not be trusted with respect to in vivo functionality (Wasserman and Sandelin, 2004), patterns of over- or underrepresentations of TF binding sites in sets of gene promoters might reflect true functionality (Frith et al., 2004). Drawing upon these lessons, we proceeded to search for pairs of predicted CMs overrepresented in promoters of 12 hr genes but not in the promoters of circadian genes. As mentioned above, we have 38 different CMs, and we classified each corresponding circadian TF as either an activator or a repressor of gene expression (Extended Results; Table S1; and references therein). For seven of the circadian TFs (e.g., YY1), the roles as activators or repressors are highly ambiguous or context dependent; hence, these TFs and CMs were excluded

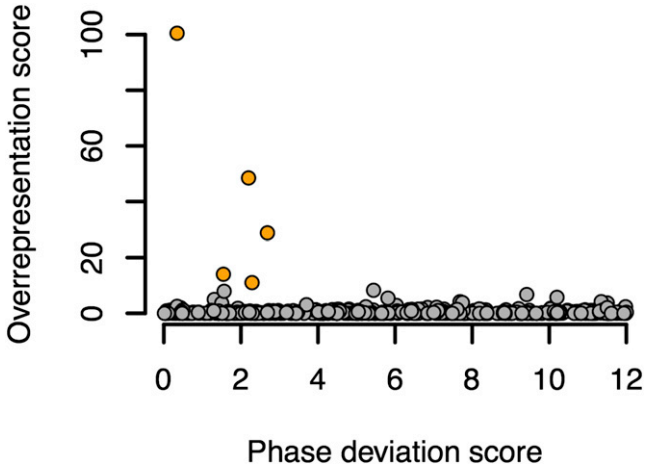


Figure 5. Overrepresented CM Pairs in Promoters of 12 Hr Genes Have Phases Conducive to 12 Hr Rhythm Generation

The overrepresentation (OR) scores of all 435 CM pairs are plotted against their phase deviation (PD) scores. The five CM pairs with an overrepresentation score larger than ten are highlighted in orange. For these pairs, the p value of their occurrence among 12 hr gene promoters is more than ten times lower than that for their occurrence among circadian gene promoters. All of these five pairs have a phase PD score smaller than three. This means that their phase differences deviate by less than 3 hr from the phase optimal for 12 hr rhythm generation as predicted by theory. As a comparison, only 119 out of all 465 CM pairs have a PD score less than 3 hr. See Table S2 for details of the top-five CM pairs. See also Figure S2 and Tables S1, S2, and S3.

from further analysis. We then have 31 different CMs, which makes for 31 × 30/2 = 465 possible pairs of CMs constituting circadian AND funnels. We computed a score for overrepresentation of CM pairs in the promoters of 12 hr genes compared to in the promoter circadian genes (Figure 3; Experimental Procedures; Extended Results), resulting in a ranked list of overrepresentation scores. To obtain further statistics, we assigned a phase deviation score to each CM pair. This score measures the deviation of a CM pair from optimal 12 hr rhythm generation using the estimated circadian phase of each TF and its classification as activator or repressor (see Experimental Procedures). A phase deviation score of 0 is optimal for 12 hr rhythm generation, whereas the maximal deviation score of 12 corresponds to the generation of a high-amplitude purely circadian rhythm. Out of the 465 CM pairs, 119 (or 26%) had a phase deviation score smaller than 3 hr. When plotting the overrepresentation score against the deviation score (Figure 5), one observes that all five pairs with overrepresentation score greater than ten have a deviation score of less than 3 hr (these pairs are given in Table S2). Statistical tests confirmed the significance of this observation ($p = 0.0010$, Fisher's exact test; $p = 0.0080$, Spearman's one-sided rank correlation test; see Extended Results for details). Of the 166 promoters of 12 hr genes, 16 were annotated to contain one or more of these top-five CM pairs. As an illustration, time series of the two TFs in the CM pair with the highest overrepresentation score (*Junb* and *Nr2f2*) together with a time series of a 12 hr gene (*Hspa5*), which has a promoter annotated to contain this CM pair, are given in Figure S2. In conclusion, we find that circadian TF binding site pairs (circadian AND funnels) that are overrepresented in the promoters of 12 hr genes have their phases aligned in accordance with the theory for 12 hr rhythm generation developed in this paper.

Binding Sites for 12 Hr TFs Are Overrepresented among the Promoter 12 Hr Genes

Our analysis so far suggests a mechanism in which the core circadian clock together with extracellular circadian cues (such as feeding patterns) is responsible for 12 hr rhythmicity among some genes in the mouse liver. Obviously, the 12 hr rhythms of some genes might as well be generated by 12 hr rhythmic TFs. To investigate this possibility, we surveyed the 12 hr genes and indeed found five TFs among them (Table S3). We next searched the promoters of 12 hr genes for binding sites of these five TFs (Experimental Procedures). One of these 12 hr rhythmic TFs, GABPA, exhibited a very strong overrepresentation among the promoters of the 12 hr genes compared to the background set of circadian promoters, whereas another 12 hr rhythmic TF, CREB3, exhibited a moderate overrepresentation (Table S3). Specifically, a GABPA binding site was detected in 97 (58%) of the promoters of 12 hr genes. Among the promoters of the circadian genes, on the other hand, only 33% of the promoters had this binding site annotated in SwissRegulon. This overrepresentation is highly significant ($p < 10^{-7}$, Fisher's exact test, corrected for multiple testing; see Extended Results). Interestingly, the promoter of GABPA contains four of the five top-scoring CM pairs identified as reported above. This is consistent with a layered rhythm generation design, in which pairs of circadian TFs forming circadian AND funnels drive 12 hr rhythms in the expression of some genes. Among the products of these genes are TFs whose 12 hr rhythms then are able to generate 12 hr rhythms in a further, possibly larger, set of 12 hr genes.

In summary, this suggests that the 12 hr rhythms of some genes are induced by 12 hr TFs. In turn, the 12 hr rhythms of these TFs can be produced by circadian AND funnels that act as the prime cause of 12 hr rhythms.

DISCUSSION

In this study, we proceeded from basic biophysical principles that govern gene regulation to derive the gene expression dynamics of genes regulated by more than one circadian TF. We studied the circadian OR funnel and showed that this motif always generates circadian rhythms. However, we have a possible explanation for the observed 12 hr gene expression rhythms of many mouse liver genes in the circadian AND funnel. This mechanism puts clear constraints on the TFs involved, in terms of activator or repressor activity, and of circadian phase, respectively. Drawing upon these constraints, we found support for the mechanism in terms of phase, as estimated from gene expression data, and in terms of predicted TF binding sites in the promoters of 12 hr genes. The mechanism also predicts a pattern of alternating large and small peaks in the 12 hr rhythm waveforms. In the set of 12 hr genes (Hughes et al., 2009), we indeed registered this pattern in more than half of the time courses, as opposed to 25% that would be expected by pure chance. Incidentally, such a pattern has also been recorded in the circatidal rhythms of certain animals (Wilcockson and Zhang, 2008). This pattern

strongly suggests that the circadian clock underlies the 12 hr rhythms.

Ukai-Tadenuma et al. (2011) proposed an additive model of circadian gene expression: the so-called phase vector model. Our results provide both a justification for this model, as well as a crucial extension: the appearance of 12 hr rhythms for some phase relationships. For circadian TFs competing for the same binding site, the phase vector model is correct. For circadian TFs binding to different binding sites of the same promoter, the phase vector model, although certainly often a good approximation (as for the experimental data in that study), should be modified according to Equation 3: it will always contain higher harmonics that may or may not be significant.

Solid statistics for TF binding sites can only be achieved through comparisons of sets of genes, so individual predictions must be interpreted with caution. Nevertheless, a few comments on the combination with the strongest signal (see Table S2), i.e., JunB and NR2F2 (COUP-TFII), are in order. As pointed out in the Introduction, there is evidence that both feeding (Vollmers et al., 2009) and cues from the SCN (Hughes et al., 2012) contribute to the induction of 12 hr rhythm in mouse liver. Specifically, Hughes et al. (2009) found no 12 hr rhythms in cultured hepatocytes, which suggests that many of the 12 hr rhythms are induced by external signals. The investigators found that some 12 hr genes reverted to circadian rhythms when mice were subjected to restricted feeding, whereas Vollmers et al. (2009) observed a loss of 12 hr rhythms in mice subjected to a restricted feeding schedule. These observations are consistent with feeding being one of the factors behind 12 hr rhythm generation. Because the restricted feeding does not shift the phases of both signaling components, which would result in retained 12 hr rhythms, it is tempting to speculate that the SCN, whose rhythms remain unaltered by restricted feeding (Damiola et al., 2000), is responsible for the second component in some 12 hr genes. Support for this idea was indeed found by Hughes et al. (2012). Interestingly, our top TF combination may represent circadian cues from these sources. COUP-TFII is repressed in the liver by feeding via insulin and glucose (Perilhou et al., 2008). Our phase-estimation procedure (Experimental Procedures) estimated the phase of COUP-TFII to around CT 6, which means that it hits the lowest point of expression at around CT 18, at the same time as food-derived substances hit their maximal abundance in mouse liver (Eckel-Mahan et al., 2012). The other TF rhythm, in JunB, may originate from the SCN. It is known that SCN-governed glucocorticoid signaling peaks in the early night (CT 14–18) for nocturnal animals (Dickmeis, 2009) and, further, that it inhibits hepatic JunB expression (Youssef and Badr, 2003). In turn, the phase of JunB is CT 6, which is antiphase to the glucocorticoid signal, consistent with SCN-induced glucocorticoid signaling inhibiting the expression of JunB.

In this study, we took a statistical, genome-wide approach to find evidence for the proposed mechanism for the generation of 12 hr rhythms. Complementary to this, it will be interesting to validate the model for a specific gene, using, e.g., ChIP to detect TF binding. The protein GABPA was predicted to be a 12 hr TF inducing 12 hr rhythms in other genes. Consequently, it would be interesting to investigate the effects of a liver-specific knockout of this gene. A muscle-specific knockout mouse has successfully been created by Jaworski et al. (2007).

The network of circadian TFs is layered. TFs of the core circadian clock induce rhythmic gene expression of target genes, some of which may be TFs themselves. The latter can be considered secondary circadian TFs. This layered network has been called the circadian transcriptional cascade (Balsalobre, 2002; Gachon et al., 2004) (Figure 1A). We argue that some branches of this cascade lead to 12 hr gene expression rhythms. These branches can be combinations of TFs of the circadian subsystem, of external cues originating from the SCN and feeding patterns, or both. The circadian subsystem of the core circadian clock can use both of the basic circadian activity motifs considered here (AND and OR funnels). The branches responsible for 12 hr rhythm generation as conceived here must use the AND funnel circadian activity motif (Figure 1A). It is possible that some of the 12 hr rhythmic genes arising in these branches themselves are TFs and, in turn, induce 12 hr rhythms in their transcriptional targets, through either AND or OR funnels. In line with this, we found five 12 hr TFs, two of which were overrepresented among the promoters of 12 hr genes, as reported above.

Our analysis also showed that if an AND funnel consists of one circadian and one 12 hr TF, then the resulting rhythms contain an additional 8 hr component. Such 8 hr rhythms were observed by Hughes et al. (2009), but only in around 60 genes. This makes sense, considering that 12 hr rhythms are scarcer than circadian rhythms among TFs. The probability of a binding site for a 12 hr TF coinciding with a binding site for a circadian TF on a promoter is lower than for two circadian TF binding sites.

It is possible that 12 hr rhythms of some genes are directly induced by extracellular 12 hr rhythms. For instance, Vollmers et al. (2009) observed 12 hr rhythms in the TF ATF6 and some of its targets. Because ATF6 is induced by feeding, these rhythms could reflect weakly bimodal feeding patterns. This was attributed to the rapid kinetics of ATF6 in response to feeding. Some 12 hr rhythms in the data set analyzed here could be induced by feeding as well. Contradicting this, however, is the fact that ATF6 was not identified as a 12 hr, nor circadian, gene in the data set we worked with in this study. Also, in a more recent study by Eckel-Mahan et al. (2012), feeding rhythms were purely circadian. Finally, we note that most feeding-induced transcripts found in the study of Vollmers et al. (2009) were classified as having circadian, not 12 hr, rhythms. Not surprisingly, then Cretenet et al. (2010) found that the 12 hr rhythms in the ER stress pathway persisted in mouse liver under food deprivation.

The mechanism proposed in this paper is not the single possible one for generation of 12 hr rhythms in mRNA abundance. Here, we focused on the perhaps simplest possible scenario, where two different circadian TFs regulate a single gene, to produce 12 hr rhythms. However, it is easy to imagine a situation in which a circadian TF and a circadian coregulator interact to produce a 12 hr gene regulation rhythm. This is because TFs and coregulators often dimerize. The concentration of the dimer will be proportional to the product of the concentrations of TF and coregulator, and as demonstrated here, this multiplicative effect is precisely what is needed to generate a 12 hr rhythm out of circadian components. Moreover, it was suggested by Hughes et al. (2009) that if not only the gene expression rate

but also the mRNA degradation rate were rhythmic, 12 hr mRNA rhythms would ensue. However, this is not what one would expect, according to standard models for mRNA degradation (S. Lueck, K. Thurley, P. Thaben, and P.O.W., unpublished data).

The analysis in the present paper has some bearing on circatidal clocks, mainly studied in crustaceans. In particular, the "circalunidian clock hypothesis" posits that the 12.4 hr circatidal rhythm is generated by two circalunidian clocks (following the lunar day of 24.8 hr) running in antiphase (Wilcockson and Zhang, 2008). We have shown here that one single-cellular circadian clock can generate 12 hr rhythms in gene expression, if outputs are split and phase shifted, or mixed in terms of activators and repressors. This possibility could be considered also in the circatidal clock field. It is possible that the circatidal rhythms observed in constant conditions are generated by genetic circadian clock circuits according to the principles outlined here. This theory is more consistent with a circatidal clock having evolved from (and probably coexisting with) circadian clocks, than the reverse scenario.

The proposed mechanism is from an evolutionary point of view easy to develop. With a functioning genetic circadian clock in place, a few point mutations in the rapidly evolving promoter regions are theoretically enough to generate a pair of TF binding sites conducive to 12 hr rhythms. In the same way, tissue specificity can be achieved by switching on or off potentially 12 hr rhythm-generating circadian TFs.

EXPERIMENTAL PROCEDURES

Our workflow for the promoter analysis and for the estimation of circadian phase of TFs is summarized in Figure 3.

Gene Expression Data

Using the liver gene expression data set of Hughes et al. (2009), as obtained from the Gene Expression Omnibus (accession number GSE11923), we generated time series corresponding to all mouse Ensembl genes as described by Durinck et al. (2009) and singled out 2,728 circadian genes and 197 12 hr genes according to the rigorous multiple testing procedure as given in the original study by Hughes et al. (2009). The time series consists of 48 time points, corresponding to CT 18 –65. For each circadian gene, we estimated its circadian phase, given in CT, by harmonic regression (cosinor analysis; see Extended Experimental Procedures for details).

Peak Height Detection

Each of the 197 time courses for 12 hr genes was subdivided into four 12-hr-long segments. The maximal gene expression level was recorded for each gene and segment. Between the four successive maxima for each time course, three peak-to-peak lines can be drawn (e.g., the dashed gray lines in Figure 2B). An alternating peak height pattern was defined based on the sign of the slopes (i.e., positive or negative) of the three straight peak-to-peak lines. There are eight different sign combinations, and two of these have an alternating pattern: the positive-negative-positive and negative-positive-negative slope successions. Thus, for random peak height successions, there is a 25% probability of an alternating pattern. This was used as the null hypothesis for the binomial test.

TF Binding Site Predictions

TF binding site predictions for the mouse genome were extracted from the SwissRegulon database (Pachkov et al., 2007). The advantage of this database is that the power of the approach taken for predicting binding sites was confirmed in a large study by Suzuki et al. (2009). These predictions are based on 180 different position weight matrices representing DNA sequence motifs for TF binding sites of 340 different TFs. Predictions were made for promoter sequences −300 to +100 bp around 19,622 mouse gene transcription start sites (TSSs) as annotated in the RefSeq database. We were able to unambiguously map 2,211 circadianly oscillating microarray probe sets and 166 12 hr oscillating probe sets, respectively, in the expression data set of Hughes et al. (2009) to RefSeq TSSs annotated in SwissRegulon. For each RefSeq TSS, we scanned which of its TF binding sites predicted in SwissRegulon belong to our list of CMs. For each RefSeq TSS, we thus obtained a list of predicted CMs.

Circadian TF Pair Overrepresentation and Phase Deviation Scores

We used the cumulative hypergeometric distribution, as previously described by Sudarsanam et al. (2002), to calculate a p value for the observed occurrence count of each possible CM pair in the set of 166 12 hr promoters, given the abundance among these promoters for each of the 31 different CMs. An occurrence of a CM pair in a SwissRegulon promoter was defined as at least one nonoverlapping coincidence of the involved SwissRegulon DNA sequence motifs in the promoter. In the same way, we calculated corresponding p values for the CM pair occurrences we observed among the 2,211 circadian promoters. For each pair, we formed a ratio of its 12 hr promoter p value to its circadian promoter p value. A small ratio thus suggests that binding sites for a given pair of CMs are more likely to occur in the same promoter among the 12 hr genes, compared to circadian genes, given the abundances of the involved CMs among the 12 hr and circadian genes, respectively. Using the circadian genes as the background set eliminates pairs that tend to occur together for other reasons than 12 hr rhythm generation. This also has the beneficial side effect of excluding occurrence artifacts due to sequence similarity within pairs (this was also separately confirmed; see Extended Experimental Procedures). We subdivided the circadian TFs into activators and repressors in a comprehensive literature study (Table S1). As mentioned, there are good reasons to expect the phase difference between a given pair of actual TFs to follow that of their transcripts (see Extended Experimental Procedures). Thus, because we are only interested in phase differences rather than absolute phases, the phase of each TF transcript (as calculated from microarray data; see above) was assigned to the corresponding CM. For the seven CMs regulated by several TFs, we defined the phases of the CMs as the circular mean of the phases of the corresponding TFs. With information of activator and repressor activity, together with the phases, we calculated a phase deviation score *PD*, which measures the deviation from the phase difference optimal for generating 12 hr rhythms for each pair, according to $PD = 12 - \Delta CT$ hours for either two activators or two repressors, and $PD = \Delta CT$ for a combination of an activator and a repressor. Here, ΔCT is the (minimal) circular distance, measured in hours, between the circadian phases of the two TFs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, three tables, Extended Results, and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.03.013.

LICENSING INFORMATION

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