

# Proof-by-synthesis of the transcriptional logic of mammalian circadian clocks

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**Mammalian circadian clocks consist of complex regulatory loops mediated through—at least—morning, daytime and night-time DNA elements. To prove the transcriptional logic of mammalian clocks, we developed an *in cellulo* mammalian cell-culture system that allowed us to design and implement artificial transcriptional circuits. Here we show that morning activation and night-time repression can yield the transcriptional output during the daytime, and similarly that daytime activation and morning repression can yield night-time transcriptional output. We also observed that the diverse transcriptional outputs of other phases can be generated through the expression of simple combinations of transcriptional activators and repressors. These results reveal design principles not only for understanding the continuous transcriptional outputs observed *in vivo* but also for the logical construction of artificial promoters working at novel phases. Logical synthesis of artificial circuits, with an identified structure and observed dynamics, provides an alternative strategy applicable to the investigation of complex biological systems.**

The transcriptional circuit underlying mammalian circadian clocks consists of at least three clock-controlled DNA elements (CCEs): morning (E-box/E<sup>-</sup>-box, CACGT[G/T])<sup>1–4</sup>, daytime (D-box, TTA[T/C]GTAA)<sup>4,5</sup> and night-time (RRE, [A/T]A[A/T]NT[A/G]GGTCA)<sup>6–8</sup>, and at least 20 clock or clock-controlled genes<sup>4,7,9–28</sup> (Fig. 1a). E-box-mediated transcription is directly or indirectly controlled by at least 11 transcription factors<sup>9–21</sup> and thus has a critical function as the core of mammalian circadian clocks<sup>4,29</sup>, whereas the D-box-mediated and RRE-mediated transcriptions are controlled by four<sup>9,24–26</sup> and five<sup>4,7,9,27,28</sup> transcription factors, respectively, and function primarily as output or stabilizing loops<sup>4,7,9,25–27</sup> of mammalian circadian clocks.

Although the network structure of these *cis*-elements and *trans*-regulators has been described comprehensively<sup>4</sup>, the dynamic principles governing this transcriptional circuit remain elusive. With an eye towards gaining a system-level understanding of the transcriptional logic underlying mammalian circadian clocks, we carefully reviewed its network structure (Fig. 1a). First, we examined the daytime expression mediated through the D-box, which is bound competitively by its transcriptional activators (*Dbp*, *Tef* and *Hlf*) and its transcriptional repressor (*E4bp4*). *Dbp*, *Tef* and *Hlf* are expressed during the morning under the control of the E-box, whereas *E4bp4* is expressed during the night-time under the control of the RRE<sup>4</sup>. On the basis of this circuit information, we formed the hypothesis that one (or more) morning activator(s) and a night-time repressor could together determine the daytime transcriptional output mediated through the D-box. Similarly, we examined the night-time

expression mediated through the RRE, which is bound competitively by its activators (*Rora*, *Rorβ* and *Rory*) and its repressors (*RevErbAα* and *RevErbAβ*). Because two of the three RRE activators (*Rora* and *Rorβ*) are expressed during the daytime under the control of the D-box, and because the RRE repressors (*RevErbAα* and *RevErbAβ*) seem strongly influenced by the two morning elements (E-boxes) in their intronic promoter regions for their morning expression, we formulated another simple hypothesis: one or more daytime activators and one or more morning repressors can specify the night-time transcriptional output by regulating the RRE. As an initial investigation into the dynamic principles governing circadian transcriptional circuits, we focused on the daytime and night-time expression among three timings in mammalian circadian clocks. To test the hypotheses derived above (from examining the natural circadian circuit) we synthesized artificial transcriptional circuits to simulate the natural circuits physically and then observed the resulting system dynamics.

## RESULTS

### *In cellulo* mammalian cell-culture system for physical simulation of natural transcriptional circuits

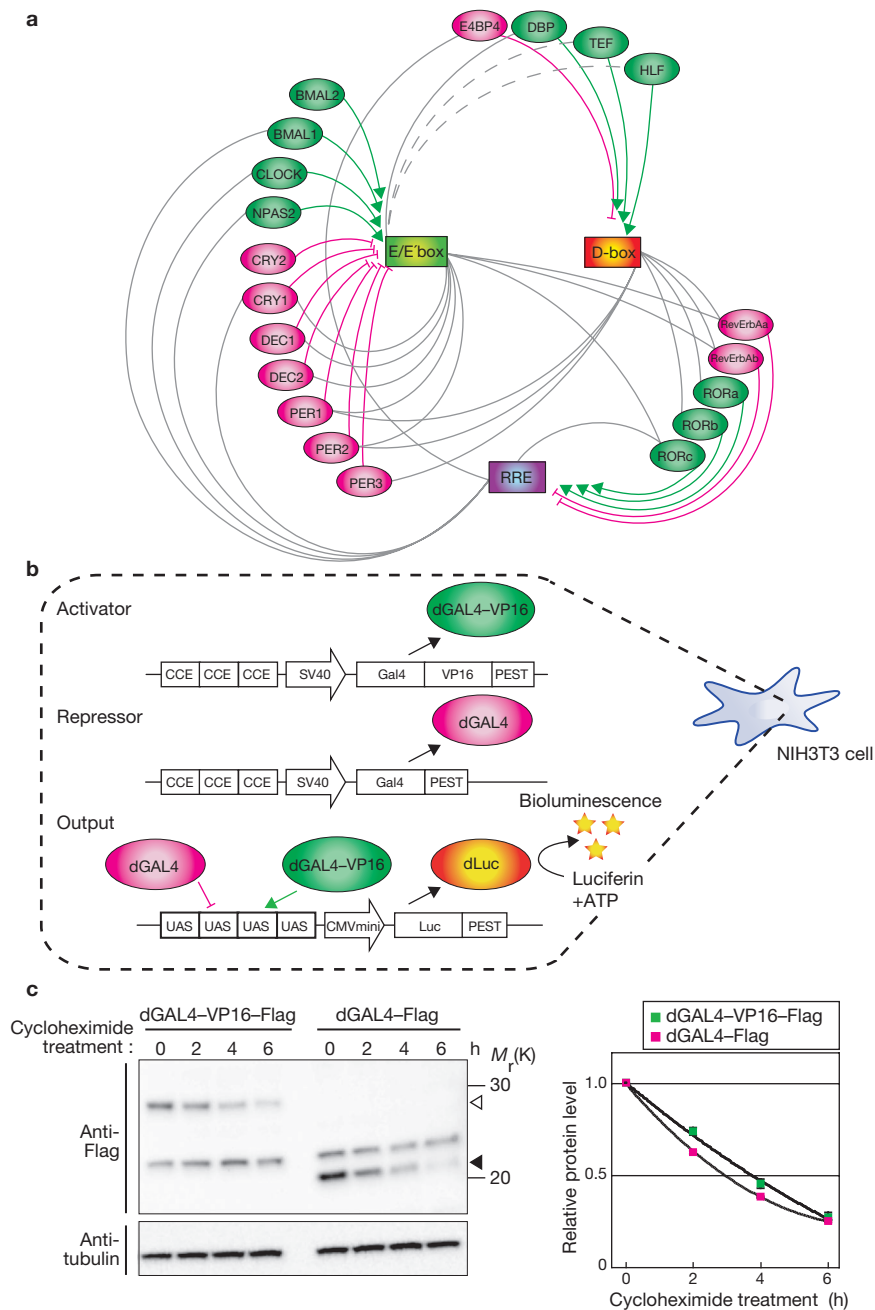
We developed an *in cellulo* mammalian cell-culture system consisting of mouse clock cells (NIH3T3 (ref. 30)), an artificial activator (destabilized GAL4–VP16 fusion proteins, dGAL4–VP16), an artificial repressor (destabilized GAL4 proteins, dGAL4), and an output reporter harbouring a gene encoding destabilized luciferase (*dLuc*) driven by a minimal cytomegalovirus promoter (CMVmini) fused with

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**Figure 1** Using an *in cellulo* mammalian cell-culture system for the physical simulation of natural transcriptional circuits. **(a)** The natural transcriptional circuit of mammalian circadian clocks. Clock-controlled elements (CCE; rectangles), transcriptional activators (green ovals) and repressors (magenta ovals) are indicated. Grey lines between CCEs and transcription factors indicate that the evolutionarily conserved CCEs are located on the promoter (or enhancer) regions of the respective transcription factor genes (grey dotted lines indicate putative CCEs determined by bioinformatics analysis). Green lines from transcriptional activators to CCEs indicate activation; magenta lines from transcriptional repressors to CCEs indicate repression. **(b)** The artificial transcriptional system synthesized in this study. A destabilized (that is, PEST-fused) GAL4–VP16 fusion protein (dGAL4–VP16) and destabilized GAL4 protein (dGAL4) were used as the

activator and repressor, respectively (top and middle). These transcription factors are expressed under the control of three tandem repeats of clock-controlled (DNA) elements (CCEs). Once expressed, the artificial activator and repressor competitively bind the four tandem repeats of the galactose UAS in the artificial promoter to regulate the output reporter gene, encoding destabilized luciferase (*dLuc*, bottom). SV40, SV40 promoter; CMVmini, minimal CMV promoter. **(c)** Western blot analysis by cycloheximide treatment experiments revealed the protein half-life of dGAL4–VP16–Flag or dGAL4–Flag. The levels of dGAL4–VP16–Flag (white arrowhead) and dGAL4–Flag (black arrowhead) proteins were normalized by using tubulin- $\alpha$  protein levels as a loading control (left panel). Quantitative densitometric analysis shows the comparable half-lives of dGAL4–VP16–Flag and dGAL4–Flag to be 3.76 h and 2.96 h, respectively (right panel). The protein levels are shown relative to  $t = 0$ .

four tandem repeats of the galactose upstream activating sequence (UAS; Supplementary Text and Supplementary Fig. S1a), which are bound by the GAL4 DNA-binding domain (Fig. 1b). This system is

designed on the basis of a previous finding<sup>31</sup> that the hybrid protein GAL4–VP16 can activate transcription efficiently in mammalian cells but the GAL4 protein alone cannot. We found that the half-lives of

dGAL4–VP16 and dGAL4 were comparable in our system: 3.76 h and 2.96 h, respectively (Fig. 1c). Thus, by extension, the artificial activator and repressor can competitively regulate the output reporter through the UAS. Finally, the artificial activator and repressor are driven by the SV40 basic promoter fused with three tandem repeats of CCEs (Supplementary Text and Supplementary Fig. S1b). We control the expression of these artificial regulators by using either the morning (E'-box), the daytime (D-box) or the night-time (RRE) elements as the CCEs<sup>4</sup>. To observe the dynamic behaviour of this artificial circuit, NIH3T3 host cells were transiently transfected with activator, repressor and reporter plasmids, stimulated with forskolin to synchronize the circadian rhythmicity of the individual cellular clocks, and then monitored in real time for bioluminescence over several days.

We reasoned that if (and only if) the peak timing of the expression (that is, the 'phase') of the transcriptional activator(s) and repressor(s) is a good determinant of the phase of the downstream transcriptional output, it should be possible to generate the natural phases in the clock cells by using synthetic transcription factors and promoters. If so, the finding would be remarkable because transcription factors are regulated by various post-transcriptional mechanisms<sup>32</sup>, including translation<sup>33</sup>, phosphorylation<sup>9,22,34–38</sup>, ubiquitylation<sup>9,38–40</sup>, sumoylation<sup>41</sup> and nuclear transportation<sup>9,40,42</sup>—these post-transcriptional mechanisms are thought to contribute, at least in part, to the phases of the downstream transcriptional outputs. In other words, a successful reconstruction of the natural circadian phases would imply that the network structure of transcriptional circuits determines the fundamental timing of the outputs from the circadian system. After transcription, the system output timing can then be reinforced or modified by the various post-transcriptional mechanisms listed above.

### Proof-by-synthesis of daytime and night-time transcriptional regulations

Using the artificial circuit system (Fig. 1b), we attempted to test our hypothesis for daytime output by examining the dynamic behaviour of the transcriptional output generated by an artificial morning activator and an artificial night-time repressor, which were controlled by E'-box elements and RRE elements, respectively. We first confirmed the circadian oscillations of output *dLuc* messenger RNA (mRNA), as well as the timing of the binding of the activator (dGAL4–VP16) and repressor (dGAL4) to the UAS—the peak times were at 2.0 h, 3.0 h and 16.0 h before the second peak of output luciferase activity, respectively (Fig. 2a, b, and Supplementary Fig. S1c). We noted that the peak time of output mRNA abundance closely (within about 1.0 h) matched the peak time of the activator binding in our *in cellulo* system. Natural circadian systems show similar timing between the peak times of mRNA output and activator binding to the DNA element—at least for E-box activators (BMAL1/CLOCK)—where the peak time of E-box-controlled mRNA expression is at zeitgeber time (ZT) 7 (ref. 43) which is in the middle of the range of peak time binding of BMAL1/CLOCK to the E-box, ZT 6–8.

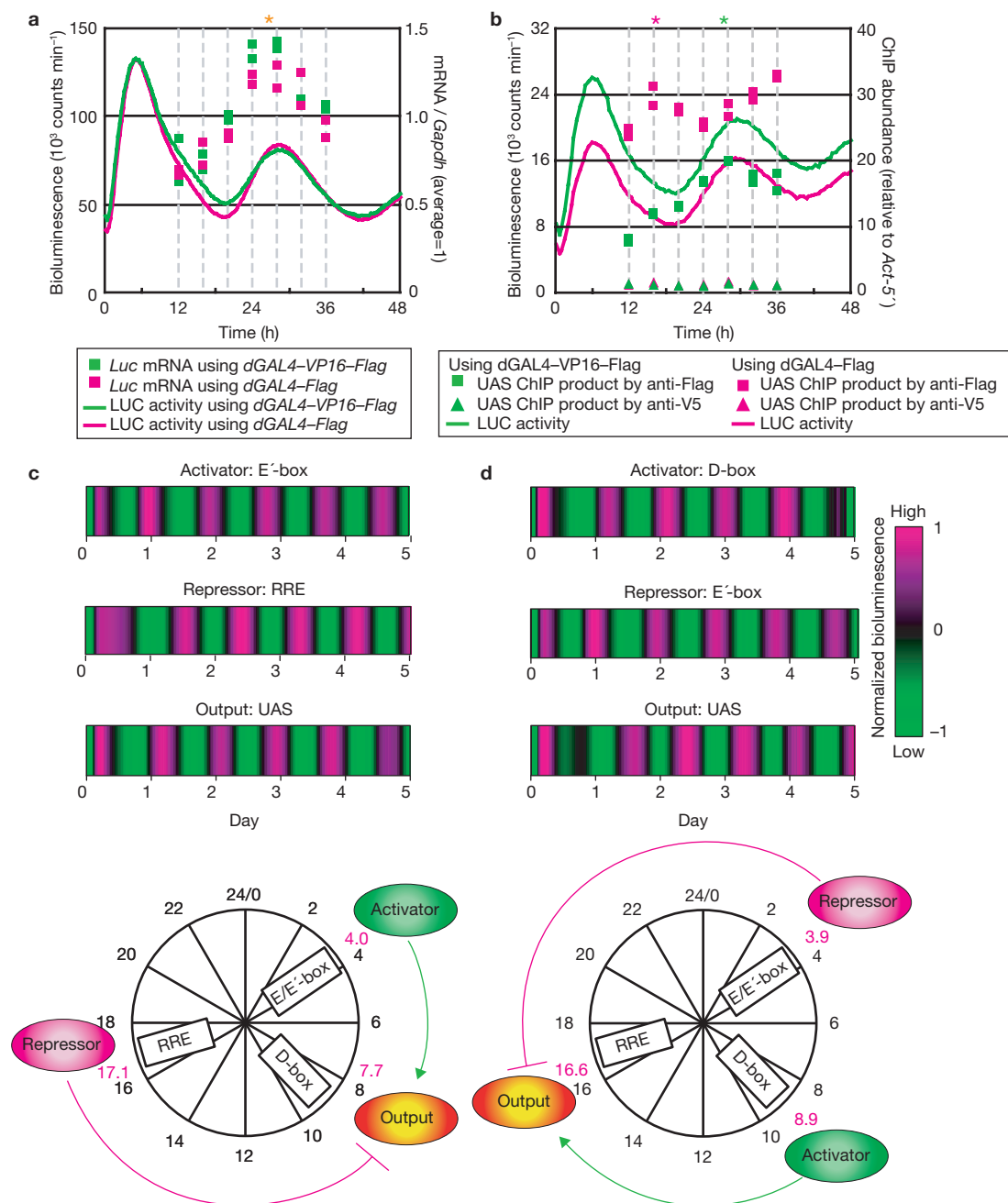
When monitored by luciferase activity, the phases of the activator and repressor in this experiment were detected at circadian time (CT)  $4.0 \pm 0.29$  (s.d.,  $n = 3$ ), and CT  $17.1 \pm 0.37$  ( $n = 3$ ; Fig. 2c and Supplementary Fig. S2a, left). The transcriptional output driven by these regulators showed circadian oscillation with a phase at CT  $7.7 \pm 0.85$  ( $n = 4$ ), which is close (1.0 h or less) to the corresponding natural daytime (D-box) phase, CT  $8.7 \pm 1.13$ . This result suggests that morning

activation and night-time repression are sufficient to determine the daytime transcriptional output. High-amplitude circadian oscillation was not detected by expressing either the morning activator or the night-time repressor alone (Supplementary Fig. S2b). Even when we used the thymidine kinase (TK) promoter fused with  $3 \times$  CCE as a weaker driver, which generates sevenfold less expression than the SV40 promoter ( $1:(7.28 \pm 0.70)$ ; Supplementary Fig. S2b, left), we still could not detect high-amplitude circadian oscillations (Supplementary Fig. S2b). These results illustrate that the competition between a morning activator and night-time repressor is essential for generating detectable circadian oscillations during daytime, which confirms observations of natural circadian systems, in which a morning activator (*Dbp*, *Tef* or *Hlf*) and a night-time repressor (*E4bp4*) also competitively bind to the D-box element<sup>9,24–26</sup>. Through this competitive binding, we speculate that the D-box element can integrate both the morning-phase information conferred by its activator (*Dbp*, *Tef* or *Hlf*) and the night-time-phase information conferred by its repressor (*E4bp4*) to generate the daytime information.

We next tested the hypothesis for night-time output by examining another artificial circuit, consisting of an artificial daytime activator under D-box control and a morning repressor under E'-box control (Fig. 2d and Supplementary Fig. S2a, right). When monitored by luciferase activity, the phases of a transcriptional activator and repressor in this experiment were at CT 8.7 and 9.1 (mean = 8.9,  $n = 2$ ) and CT 3.8 and 4.0 (mean = 3.9,  $n = 2$ ), respectively. The transcriptional output driven by the regulators showed circadian oscillation with its phase at CT  $16.6 \pm 1.04$  ( $n = 3$ ), which is also very close (1.0 h or less) to the corresponding natural night-time phase (CT  $17.0 \pm 0.81$ ). As with the daytime transcriptional output, a high-amplitude circadian oscillation could not be generated by expressing the daytime activator or morning repressor alone (Supplementary Fig. S2b). These results therefore also confirmed that the competition between a daytime activator and a morning repressor is important in generating detectable circadian oscillation during the night-time in cells. Together, our reconstruction experiments for daytime and night-time expressions imply that the input phases of transcription factors can determine the phases of transcriptional output.

### Synthesis of novel output phases *in cellulo* and *in vivo*

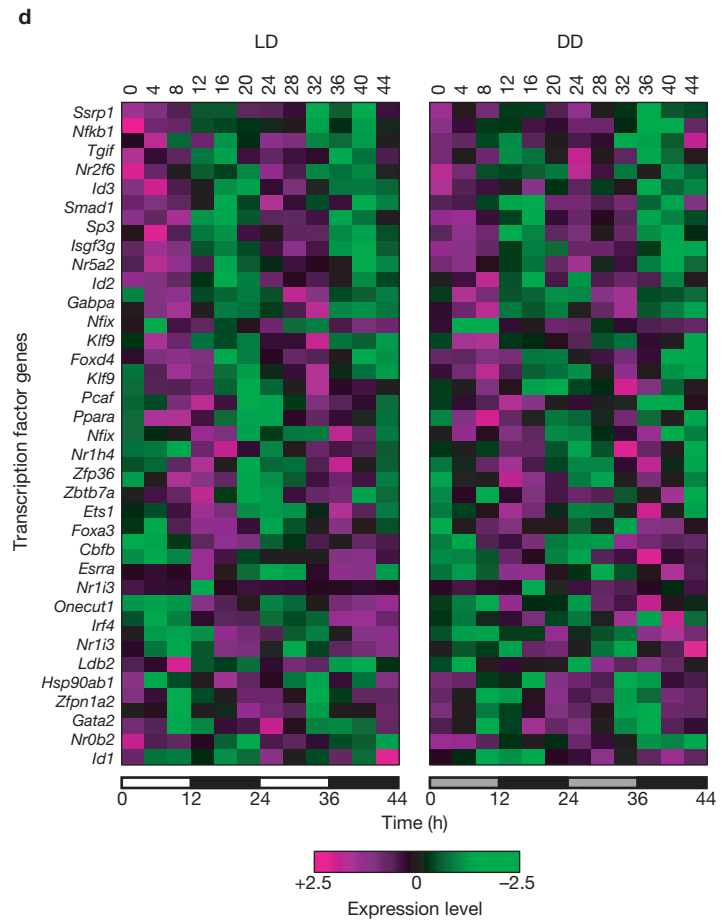
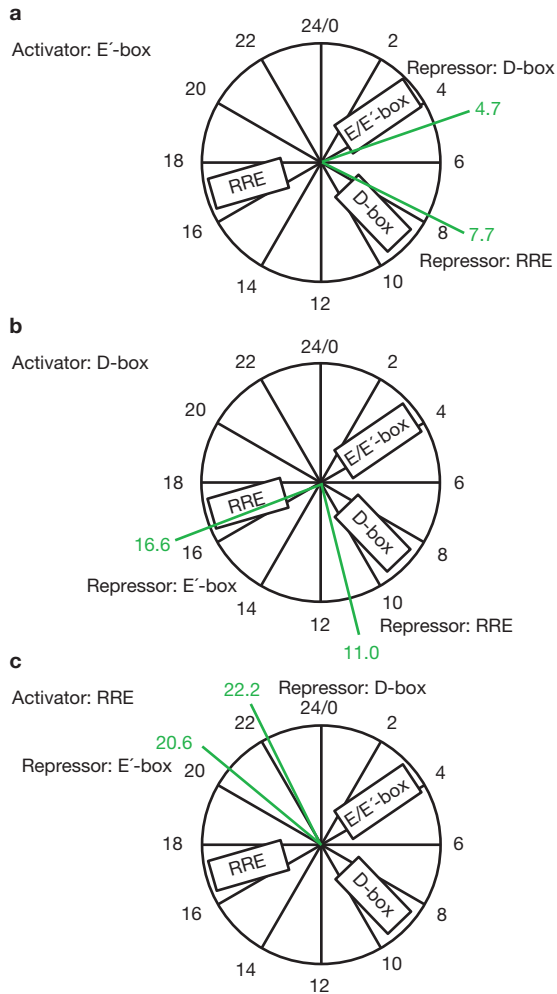
These findings led us to reason that various combinations of transcription factors with CCEs for the three basic circadian phases (morning, daytime and night-time) might generate not only the basic phases, such as daytime and night-time, but also other phases. To explore this possibility, we examined artificial circuits with various other activator–repressor pairs. In a circuit with an artificial night-time activator under RRE control and a daytime repressor under D-box control, we observed high-amplitude circadian output with the phase at CT  $22.2 \pm 0.70$  ( $n = 4$ ), which is 1.8 h before a subjective dawn (Fig. 3a and Supplementary Fig. S2c, left). We then tested the remaining combinations of regulators: that is, a night-time activator and a morning repressor, a morning activator and a daytime repressor, and a daytime activator and a night-time repressor. All of these artificial circuits showed circadian transcriptional outputs, with phases at CT  $20.6 \pm 0.91$  (s.d.,  $n = 4$ ; a subjective late night), CT  $4.7 \pm 0.40$  (s.d.,  $n = 3$ ; 1.3 h before a subjective noon) and CT  $11.0 \pm 0.79$  (s.d.,  $n = 3$ ; 1.0 h before a subjective dusk), respectively (Fig. 3b–d and Supplementary Fig. S2c). From these results we concluded that, through simple combinations of the transcription factors of the three basic circadian phases, we can generate diverse transcriptional outputs that peak close to a subjective noon, dawn, dusk or late night.



**Figure 2** Proof-by-synthesis of daytime and night-time transcriptional regulations. **(a)** Quantification of the output *dLuc* mRNA from the artificial output reporter. The amounts of *dLuc* mRNA relative to constitutively expressed *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) mRNA (points) are indicated along with the luciferase (LUC) activity (lines) for two different artificial transcriptional circuits: one using *dGAL4-VP16-Flag* (green) and another using *dGal4-Flag* (magenta). The estimated peak of output *dLuc* mRNA (marked with an orange asterisk) occurs 2.0 h before the second peak of output luciferase activity. **(b)** Quantification of GAL4-VP16-Flag and GAL4-Flag bindings to the UAS in artificial transcriptional circuits. After normalization for the amount of input DNA, the amounts of UAS regions immunoprecipitated by anti-Flag antibody (squares) and by anti-V5 antibody (triangles) were quantified relative to a constitutively unbound *actin* promoter region (*Act-5'*). These relative amounts of UAS ChIP products are indicated along with the luciferase activity (line) for two different artificial transcriptional circuits using *dGAL4-VP16-Flag* (green) and *dGAL4-Flag* (magenta) (see also Supplementary Fig. S1c for other results of the negative

control). The estimated peak times of UAS ChIP products bound by the activator (*dGAL4-VP16*, marked with a green asterisk) and the repressor (*dGAL4*, marked with a magenta asterisk) were 3.0 and 16.0 h before the second peak of output luciferase activity, respectively. **(c, d)** Synthesis of artificial transcriptional circuits: morning activator under E'-box control and night-time repressor under RRE control (c), and daytime activator under D-box control and morning repressor under E'-box control (d). Heat maps (top) indicate high (magenta) or low (green) representative promoter activities and output of each artificial transcriptional circuit monitored by bioluminescence from NIH3T3 cells. The bioluminescence data were detrended in baseline and amplitude, then normalized to set their maximum, minimum and average to 1, -1 and 0, respectively (see also Supplementary Fig. S2a for the raw bioluminescence data). The schemes at the bottom summarize the timings of peaks (that is, 'phases') of promoter activity, where an activator (green oval), repressor (magenta oval) and output reporter (orange oval) are indicated with their phases in circadian time (magenta numbers).





**Figure 4** Transcriptional mechanisms to generate various output phases. **(a–c)** Output phases in artificial transcriptional circuits with a morning activator **(a)**, daytime activator **(b)** or night-time activator **(c)**. The phase of an output reporter (green lines and numbers) is indicated in circadian time (hours) for each repressor used. In artificial transcriptional circuits *in cellulo*, the output phase could be changed according to the input phase

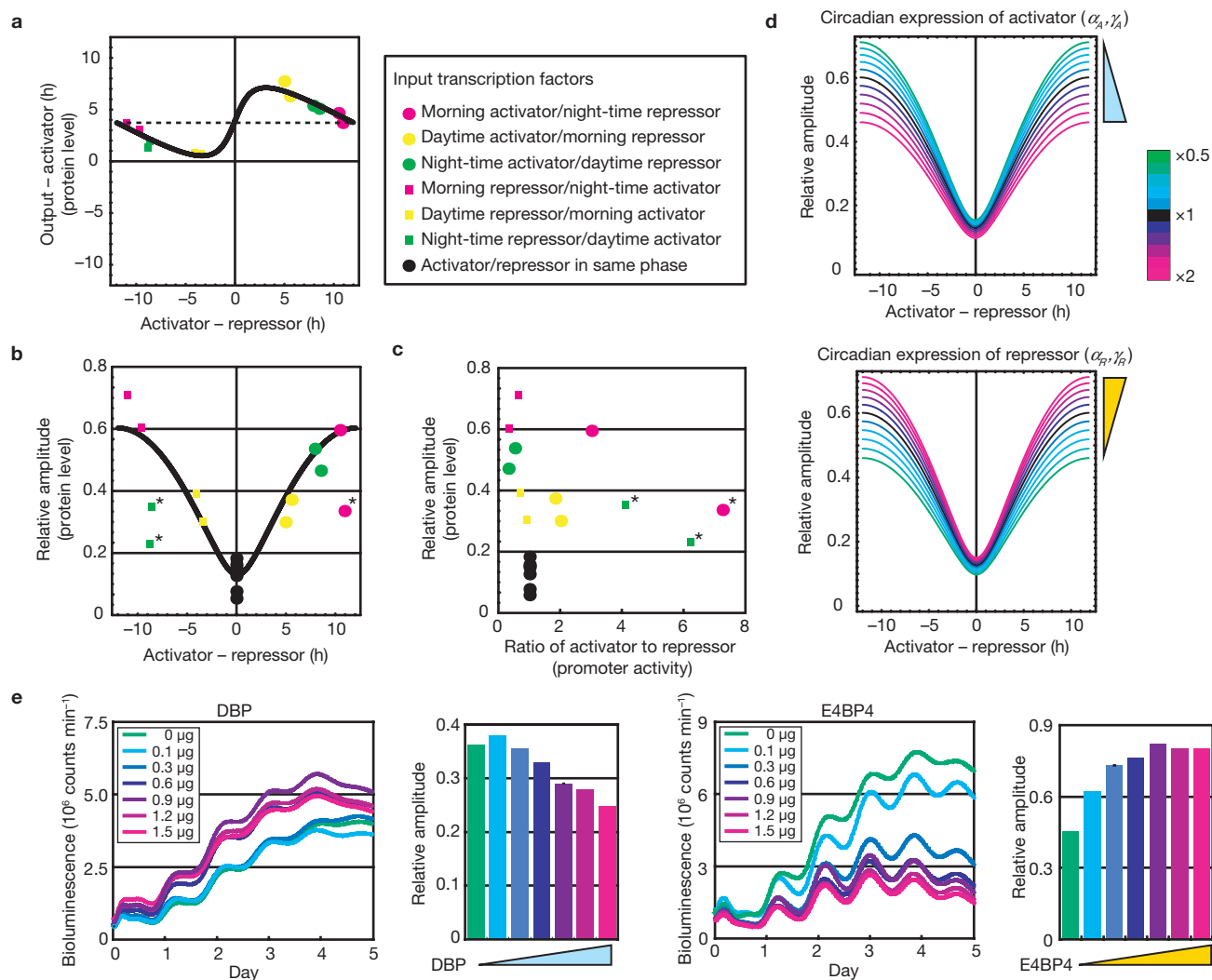
E-boxes, D-boxes or RREs (Supplementary Table S1). Furthermore, transcriptional activator(s) and repressor(s) binding to similar DNA elements are expressed at various phases *in vivo* (Supplementary Table S1), and are therefore expected, from our *in cellulo* findings, to generate additional output phases.

### Transcriptional logic underlying mammalian circadian clocks

On the basis of the findings in this study, we can now examine two predictions on the timing ('phase') and relative amplitude of circadian output oscillations in our previous report<sup>4</sup>, which proposed that the phase advancement of a repressor relative to an activator causes a phase delay of expression ('repressor-precedes-activator' mechanism), whereas the phase advancement of an activator relative to a repressor causes a phase advancement of expression ('activator-precedes-repressor' mechanism). The previous paper also proposed that a larger phase difference between an activator and a repressor generates a greater relative amplitude of output rhythm ('repressor-antiphase-to-activator' mechanism). To verify these predictions, we modelled the transcriptional system by

using an activator, a repressor and an output reporter. To implement this transcriptional system numerically, we searched for the sets of parameters that could reproduce the observed reporter output of the *in cellulo* mammalian cell-culture system (see Supplementary Text). We then plotted the theoretical relationship between the output phases (the delay/advancement of output expressions) and the input phases (the phase differences between activators and repressors; Fig. 5a) and found that the experimental data (coloured dots) matched the theoretical plot (black line). This supported the 'repressor-precedes-activator' and 'activator-precedes-repressor' mechanisms for the output phases. These repressor-precedes-activator and activator-precedes-repressor mechanisms can delay or advance the default output phase (Fig. 5a, dashed line) to generate new output phases, respectively (see also Fig. 5a, green circle and square), which probably serve to create the seemingly continuous output phases observed *in vivo*<sup>8,44–46</sup>.

We also plotted the theoretical relationship between the relative amplitudes of outputs and the input phases (the phase differences between activators and repressors; Fig. 5b). We noticed that some outliers had



**Figure 5** Transcriptional logic underlying mammalian circadian clocks. **(a)** Observed and simulated relationships between input transcription factors and the phase of the output reporter. Axes represent the phase differences (hours) between activator and repressor ( $x$  axis) and between output and activator ( $y$  axis). Points show the means of the measurement data (duplicated independently)—different point colour and shape combinations represent different transcription factor combinations. The curve shows a simulated relationship calculated from a theoretical model. The dashed line (at about +4 h) shows the default output reporter phase that occurs when a repressor is antiphasic to an activator. **(b)** Observed and simulated relationships between input transcription factors and the relative amplitude of the output reporter. The  $y$  axis represents the relative amplitude of the output reporter. The curve shows a simulated relationship calculated from our theoretical model. The points marked with an asterisk indicate outliers that have a lower relative amplitude (by 0.2 or more) than the predicted curve. **(c)** Observed relationships between the changes in expression levels of input transcription factors and the relative amplitude of the output

reporter. The  $x$  axis represents the ratio of activator expression to repressor expression. The outlier points marked with an asterisk indicate experiments in which there was a large difference (more than fourfold) in the expression levels of activator relative to repressor (the points marked with an asterisk here are consistent with the outliers in **b**). **(d)** The predicted sensitivity of the relative amplitude when the circadian expression of an activator or a repressor is changed fourfold (proportional changes in the expression level of transcription factor affect both amplitude  $\alpha$  and baseline  $\gamma$  simultaneously). Activator or repressor expression levels were varied fourfold (from  $\times 0.5$  to  $\times 2$ ); the coloured curves indicate the predicted effects of these changes in expression levels of transcription factors on relative amplitude of reporter output. **(e)** Perturbation experiments of natural transcriptional circuits. The relative amplitudes of luciferase activity are derived from the raw bioluminescence data of a daytime output reporter ( $p3 \times D\text{-box-SV40-}dLuc$ ) for various amounts of activator (DBP from  $p3 \times E'\text{-box-SV40-}Ddbp$ ) or repressor (E4BP4 from  $p3 \times RRE\text{-SV40-}E4bp4$ ). Shown are the averages of the raw bioluminescence data from two or three independent samples.

a lower relative amplitude (by 0.2 or more; Fig. 5b, asterisked) than the predicted curve (Fig. 5b, black line), which seems to have been due to the larger changes (fourfold or more) in the expression levels of activator or repressor (Fig. 5c). These results are consistent with the prediction from the sensitivity analysis of the relative amplitude and phase for a simulation model with representative parameter values (Supplementary Text and Supplementary Fig. S4), in which the increased circadian expression of

activator (simultaneous increase in both amplitude  $\alpha_A$  and baseline  $\gamma_A$ ) can decrease the relative amplitude of output oscillations (Fig. 5d), while not greatly affecting the output phase (Supplementary Fig. S4a, panels enclosed in the frame). Aside from the outliers (which are due to the greater expression of the activator), experimental data (Fig. 5b, coloured dots) matched the theoretical plot (Fig. 5b, black line), supporting the ‘repressor-antiphasic-to-activator’ mechanism for the relative amplitude.

This ‘repressor-antiphase-to-activator’ mechanism is intuitive because it probably reflects the number of hours during which the activator can activate before the repressor begins to repress. However trivial it may seem, this intuitive repressor-antiphase-to-activator mechanism is non-trivial and is even critical in generating higher-amplitude promoter activity in living cells, because no detectable oscillations are generated when a repressor is expressed in phase with an activator in various combinations (Supplementary Fig. S2d) or when an activator or repressor is expressed alone (Supplementary Fig. S2b).

### Perturbation experiments of the natural transcriptional circuit in mammalian circadian clocks

One of the benefits and meanings of theoretical analysis is its formulation of a prediction that, if verified, leads us to a deeper understanding of the system in question. As discussed above, our simulation model with representative parameter values (see Supplementary Text) shows that an increase in circadian expression of an activator or repressor causes a decrease or an increase, respectively, in the relative amplitudes of the outputs (Fig. 5d). We reasoned that if (and only if) our simulation model could recapitulate the natural circadian system, it should be possible to apply the predictions derived from our simulation model to the natural transcriptional circuit in mammalian circadian clocks. To verify our predictions, we performed two perturbation experiments to increase the circadian expression of a natural activator, DBP, or repressor, E4BP4, by using  $p3 \times E' \text{-box-SV40-Dbp}$  or  $p3 \times RRE\text{-SV40-E4bp4}$ , respectively. As predicted, the relative amplitude of a natural downstream output from the D-box element, which is monitored by  $p3 \times D\text{-box-SV40-dLuc}$ , is decreased (0.68-fold) or increased (1.76-fold) by the increase in circadian expression of activator or repressor, respectively (Fig. 5e). On the basis of these results, we concluded that both our simulation model and the derived design principles successfully recapitulated the natural transcriptional circuit in the mammalian circadian clocks.

### DISCUSSION

In this study we successfully reconstructed two natural phases of the circadian clock (that is, daytime and night-time expression). The probability that any artificially generated peak will lie within 1 h of the naturally observed output peak is 2 in 24 (or 0.083) at best. The chance that two of two experiments would produce outputs within a 1 h difference from the targets is therefore  $0.083 \times 0.083 = 0.0069$  (less than 0.05). Even if we adopt more stringent statistical criteria (the probability that two of three basic phases would be generated), the chance is (number of ways of selecting any 2 from 3)  $\times 0.083 \times 0.083 \times (1 - 0.083) = 0.0191$  (less than 0.05). On the basis of these calculations, we conclude that our reconstruction of two natural phases in the circadian clock is statistically significant.

In this study we focused mainly on the competitive binding between transcription activators and repressors that control the phase and amplitude of the circadian output oscillations in mammalian circadian clocks. However, in the natural circadian clock systems of mammals and other organisms, transcription factors undergo various types of post-transcriptional regulation<sup>9,22,32,34–42</sup> such as modification and degradation, which may also contribute to the timing of the output phase and its amplitude. It will therefore be important in future to incorporate such post-transcriptional regulation into our *in cellulo* system to investigate its physical role in a circadian system. For example, it is already known that the VP16 polypeptide used in our *in cellulo* system is subject to ubiquitylation,

which is important in transcriptional activation<sup>47</sup>. Once the VP16-specific ubiquitin ligase has been identified (it is as yet unidentified) it can be applied to our *in cellulo* system to investigate the impact of the post-transcriptional regulation on the circadian output oscillations.

In theory, the design principles described here for the phase and relative amplitude in mammalian circadian clocks can also be applied to the post-transcriptional regulation of transcription factors in mammals and other organisms, thereby allowing one to predict whether cyclic post-transcriptional regulation—depending on its phase—advances or delays the phase of the output, and whether it increases or decreases the relative amplitude of the output. For instance, the WC-1–WC-2 activator complex is expressed rhythmically by post-transcriptional regulation, whereas the *wc-1* and *wc-2* mRNAs in the *Neurospora* clock system are expressed constantly. The WC-1–WC-2 activator complex peaking at CT 17–18 and the FRQ repressor peaking at CT 8–10 drives many clock-controlled genes, and the overt rhythm in banding peaks at about CT 22 (refs 48, 49). This is quite consistent with the behaviour of our artificial circuit, in which an artificial night-time activator under RRE control (peaking at about CT 17–18) and a daytime repressor under D-box control (peaking at about CT 8) yield a high-amplitude circadian output whose phase is at CT 22.2—about 1.8 h before subjective dawn (Fig. 3a). This consistency implies a general applicability of the transcriptional logic that we describe in this study.

In summary, this study presents a synthetic approach to the ‘proof-by-synthesis’ of transcriptional logic. This approach provides us with a new strategy, not only to investigate the sufficiency of identified components of transcriptional circuits and their interactions, but also to reveal as yet unidentified components or interactions. For example, although we successfully (re)generated two basic phases, daytime and night-time, as well as additional phases near the subjective noon, dawn, dusk and late night, from the three basic circadian phases, we have not yet been able to regenerate one basic circadian phase, morning, which we expect to be regulated directly or indirectly by the three basic phases, to maintain circadian oscillations. Thus, in our *in cellulo* study, morning transcriptional regulation is still a ‘missing link’ in the mammalian clock system. Because a strong repressor at evening phase seems indispensable to the reconstruction of the morning phase, a candidate transcription factor could be CRY1, which is a transcriptional repressor expressed during the evening<sup>4</sup>. However, the expression regulation mechanism of the *Cry1* gene remains unknown because more than one DNA element seems involved in the expression<sup>4</sup>. Hence, the challenge of synthesizing evening and morning expression still lies ahead as we work towards the complete reconstruction of the transcriptional circuits underlying mammalian circadian clocks.

### METHODS

**Construction of plasmid pG4-dLuc, p3 × CCE-SV40-dGAL4-VP16 and p3 × CCE-SV40-dGAL4.** To construct the output reporter plasmids, the pG5Luc (containing five UAS) plasmid in the CheckMate Mammalian Two-Hybrid system (Promega) was modified to contain four tandem repeats of the galactose UASs and a gene encoding destabilized luciferase (*dLuc*) as follows: pG5Luc was digested with *EcoRI* and *NheI*, blunted with T4 DNA polymerase, and ligated with itself. The product was then digested with *SphI* and *Sall*, ligated to the *SphI*–*Sall* fragment from the SV40-*dLuc* plasmid, containing the PEST sequence of *dLuc*<sup>4</sup>, and termed pG4-*dLuc*. To construct activator or repressor plasmids, we modified the pBIND and pACT (Promega) vectors as follows. We amplified the VP16 coding sequences from pACT (Promega) by PCR with a forward primer containing an *EcoRV* recognition sequence (5′-GATATCCTCGACGGCCCCCGACCG-3′) and a reverse primer containing a *NotI* recognition sequence (5′-GCGGCCGCTCTAGATGGCGATCCCGACCGGGGAATC-3′). The PCR product was then digested with *EcoRV* and *NotI*, and then fused to the *Gal4* gene in the pBIND vector.



A PEST fragment, amplified from the SV40-*dLuc* plasmid by PCR with forward (5'-ATGCGGTACCAGCCATGGCTTCCC GCCG-3') and reverse (5'-CGTGGGTACCCACATTGATCCTAGCAGAAGC-3') primers containing the *KpnI* recognition sequence, was inserted into the VP16-fused and original pBIND vectors, previously digested with *KpnI*. These products were termed pCMV-*dGAL4-VP16* and pCMV-*dGal4*, respectively. To drive the artificial activator or repressor in three basic circadian phases (morning, daytime or night-time), p3 × CCE-SV40-*dLuc* plasmids—which contained the *Per2* E'-box (5'-gcgcgcggtCACGTTtccactatgtg acagcggag-3'), the *Per3* D-box (5'-cccgcggtTATGTAAggtactcg-3') or the *Bmal1* RRE (5'-aggcagAAAGTAGGTCAgggagc-3') as a CCE (CCEs are indicated with underlines)—were digested with *KpnI* and *HindIII*, blunted by T4 DNA polymerase, and inserted into the *BglII* and *NheI* site of pCMV-*dGAL4-VP16* or pCMV-*dGal4*. The end products, p3 × CCE-SV40-*dGAL4-VP16* and p3 × CCE-SV40-*dGal4*, were used as the activator or repressor plasmids for the artificial transcription circuits.

**Real-time circadian reporter assays.** Real-time circadian assays were performed as described previously<sup>1,29</sup>, with the following modifications. NIH3T3 cells (American Type Culture Collection) were grown in DMEM medium (Invitrogen) supplemented with 10% FBS (JRH Biosciences) and antibiotics (25 U ml<sup>-1</sup> penicillin, 25 mg ml<sup>-1</sup> streptomycin; Invitrogen). At 24 h before transfection, cells were plated at 10<sup>5</sup> cells per well in 35-mm dishes. These cells were transfected with FuGene6 (Roche) in accordance with the manufacturer's instructions. The cells in each well were transfected with three plasmids (0.4 μg of *dLuc* reporter plasmid, 0.025 μg of p3 × CCE-SV40-*dGAL4-VP16* and 0.1 μg of p3 × CCE-SV40-*dGal4*). As the *dLuc* reporter plasmid we used p3 × CCE-SV40-*dLuc*<sup>4</sup> for monitoring the promoter activity of the artificial transcription factors, or pG4-*dLuc* for monitoring the transcriptional output in artificial transcriptional circuits. The amount of transfected plasmid was adjusted to 2.0 μg with empty vector. After 72 h, the medium in each well was replaced with 2 ml of culture medium (DMEM/10% FBS) supplemented with 10 mM HEPES (pH 7.2; Invitrogen), 0.1 mM luciferin (Promega), antibiotics and 0.01 μM forskolin (Nacalai Tesque). Bioluminescence was measured with photomultiplier tube detector assemblies (LM2400; Hamamatsu Photonics). The modules and cultures were maintained in a darkroom at 30 °C and interfaced with computers for continuous data acquisition. Photons were counted for 1 min at 12-min intervals.

**Rhythmicity, period length and phase analysis of real-time bioluminescence data.** The rhythmicity and period length of the promoter activity for each reporter were determined as described previously<sup>29</sup>. Bioluminescence time-series data were detrended by subtracting the trend curve of a 42 h timescale, calculated by the smoothing spline method with corresponding stiffness<sup>29</sup>, and then used in the following analysis. Autocorrelation of the detrended time-series data was then calculated within the range 16–28 h to determine the circadian period of oscillation, which was defined to provide the strongest autocorrelation. Statistical significance (with  $p = 0$  as most significant and  $p = 1$  as least significant) of the circadian oscillation was evaluated by comparing the strongest autocorrelation of the detrended data within the range 16–28 h against that of white noise ( $p < 0.01$ ). The periods of three *dLuc* reporters (two p3 × CCE-SV40-*dLuc* reporters for monitoring the promoter activity of the activator or repressor, and a pG4-*dLuc* reporter for monitoring the promoter activity of the output reporter) were calculated for each artificial circuit, and the mean value of the three periods was considered the 'circuit period'.

To calculate the normalized bioluminescence data (that is, the oscillatory component of the bioluminescence data) shown in Fig. 2c–d and Fig. 3a–d, the moving average of the absolute value of the detrended bioluminescence data was calculated first. The window size of the moving average was set to half of the circuit period calculated above. Then the oscillatory component of the detrended data was calculated by dividing the data by the moving average of the data at each time point. To calculate the peak time, a cosine curve was fitted to have maximum correlation with the normalized bioluminescence data. The peak time of the fitted cosine curve was used as the phase of each reporter.

**Calculation of natural output phases of the E'-box, the D-box and the RRE.** For comparison with the artificial output phases, the natural output phases controlled by the CCEs were determined as follows. NIH3T3 cells were transfected with p3 × CCE-SV40-*dLuc*, the *dLuc* gene being driven by the SV40 basic promoter fused with three tandem repeats of CCEs, and then monitored for 5 days for each CCE ( $n = 25$ –30). The means and standard deviations of the phase for the CCEs were  $3.8 \pm 0.84$  (E'-box),  $8.7 \pm 1.13$  (D-box) and  $17.0 \pm 0.81$  (RRE) in CT,

where we define CT 0 as the time of forskolin stimulation. These mean values were regarded as the natural output phases for the morning (E'-box), daytime (D-box) and night-time (RRE) elements in the experiments.

**GeneChip expression data.** The previously published genome-wide expression data for the liver<sup>6</sup> were re-analysed and re-annotated in this study, as described in detail in Supplementary Methods.

**Theoretical model of the circadian circuit.** Our theoretical model is based on the formula in our previous report<sup>4</sup> and has been extended to introduce basal transcription, degradation of mRNAs, and the translation and degradation of proteins to express the amount of the output proteins. The model comprises five formulas: the protein levels of the activator and repressor, the transcriptional activity of the output reporter, the mRNA level of the output reporter, and the protein level of the output reporter.

The protein levels of the activator and repressor are modelled as the sum of an oscillating component and a constant component. A formula for the protein level of an activator at time  $t$  is

$$A(t) \equiv \alpha_A \left( 1 + \cos \left( 2\pi \frac{t - \alpha}{24} \right) \right) + \gamma_A$$

where  $\alpha_A$ ,  $a$  and  $\gamma_A$  are the amplitude, phase and constant component of the activator, respectively. A formula for the protein level of a repressor at time  $t$  is

$$R(t) \equiv \alpha_R \left( 1 + \cos \left( 2\pi \frac{t - b}{24} \right) \right) + \gamma_R$$

where  $\alpha_R$ ,  $b$  and  $\gamma_R$  are the amplitude, phase and constant component of the repressor, respectively.

The transcriptional activity of an output reporter at time  $t$  is formulated as

$$T(t) \equiv V_1 \left( \frac{\left( \frac{A(t)}{K_A} \right)^n}{1 + \left( \frac{A(t)}{K_A} \right)^n + \left( \frac{R(t)}{K_R} \right)^n} + \beta \right)$$

where  $1/K_A$  and  $1/K_R$  are the strengths of the binding affinities of the activator and repressor, respectively. The constants  $K_A$  and  $K_R$  are inversely proportional to the strengths of the binding affinities (the larger the values of  $K_A$  and  $K_R$ , the weaker the binding),  $n$  is the Hill coefficient,  $\beta$  is the basal transcriptional activity and  $V_1$  is the efficiency of transcription.

The mRNA and protein levels of an output reporter,  $M(t)$  and  $P(t)$ , are modelled using differential equations, and formulated as

$$\frac{d}{dt} M(t) = T(t) - \left( \frac{\log 2}{T_1} \right) M(t)$$

$$\frac{d}{dt} P(t) = V_2 M(t) - \left( \frac{\log 2}{T_2} \right) P(t)$$

where  $V_2$  is the efficiency of translation, and  $T_1$  and  $T_2$  are the half-life of the mRNA and protein, respectively.

Detailed information of the other experimental and computational methods is described in Supplementary Methods.

*Note: Supplementary Information is available on the Nature Cell Biology website.*

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## AUTHOR CONTRIBUTIONS

H.R.U. developed the concept of the proof-by-synthesis of the transcriptional logic underlying mammalian circadian clocks. M.U. constructed all materials used in this work and performed the real-time luminescence assays. T.K. developed the data analysis methods for the *in cellulo* and *in silico* data, and conducted these analyses. All the authors discussed the results and commented on the manuscript text.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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