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Unveiling "Musica Universalis" of the Cell: A Brief History of Biological 12-Hour Rhythms

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"Musica universalis" is an ancient philosophical concept claiming the movements of celestial bodies follow mathematical equations and resonate to produce an inaudible harmony of music, and the harmonious sounds that humans make were an approximation of this larger harmony of the universe. Besides music, electromagnetic waves such as light and electric signals also are presented as harmonic resonances. Despite the seemingly universal theme of harmonic resonance in various disciplines, it was not until recently that the same harmonic resonance was discovered also to exist in biological systems. Contrary to traditional belief that a biological system is either at stead-state or cycles with a single frequency, it is now appreciated that most biological systems have no homeostatic "set point," but rather oscillate as composite rhythms consisting of superimposed oscillations. These oscillations often cycle at different harmonics of the circadian rhythm, and among these, the ~12-hour oscillation is most prevalent. In this review, we focus on these 12-hour oscillations, with special attention to their evolutionary origin, regulation, and functions in mammals, as well as their relationship to the circadian rhythm. We further discuss the potential roles of the 12-hour clock in regulating hepatic steatosis, aging, and the possibility of 12-hour clock–based chronotherapy. Finally, we posit that biological rhythms are also musica universalis: whereas the circadian rhythm is synchronized to the 24-hour light/dark cycle coinciding with the Earth's rotation, the mammalian 12-hour clock may have evolved from the circatidal clock, which is entrained by the 12-hour tidal cues orchestrated by the moon.

Freeform/Key Words: 12h-clock, aging, chronotherapy, ER stress, mitochondria, NAFLD, Xbp1

1. The Concept of Harmonics in Physics and Their Biological Counterpart

The term "harmonic" originates in musical instrument theory, where the wavelengths of the overtones of a vibrating string are derived from its fundamental wavelength. A harmonic of such a wave has a frequency that is a positive integer multiple of this fundamental frequency, which is also called the first harmonic [1, 2]. For instance, as shown in Fig. 1A, the fundamental, or the first, harmonic is a wave with a frequency of 200 Hz, whereas the second harmonic is 400 Hz (200 Hz × 2 = 400 Hz), and so on. Summing the harmonics generates a composite waveform that looks "distorted and noisy" (Fig. 1A, bottom) but is actually much closer to perception of music than the individual harmonics. This is because pure oscillation with a single frequency rarely exists in nature. Rather, physical rhythms often present as superpositions of basic waves such as the harmonic resonances found in music, light, and Keplers Law of planetary motion.

To uncover all superimposed oscillations in an unbiased manner, we used a recently developed eigenvalue/pencil method [3, 4–6] and disclosed that just as for other forms of

Abbreviations: CT, circadian time; dGFP, destabilized green fluorescence protein; *Gck*, glucokinase; ER, endoplasmic reticulum; MEF, mouse embryonic fibroblast; mtDNA, mitochondrial DNA; NAFLD, nonalcoholic fatty liver diseases; TTFL, transcriptional/translational feedback loop; UPR, unfolded protein response; UPR^{ER}, endoplasmic reticulum–associated unfolded protein response; UPR^{mt}, mitochondrial unfolded protein response; XBP1s, spliced XBP1; Xbp1 us, unspliced Xbp1; ZT, Zeitgeber time.

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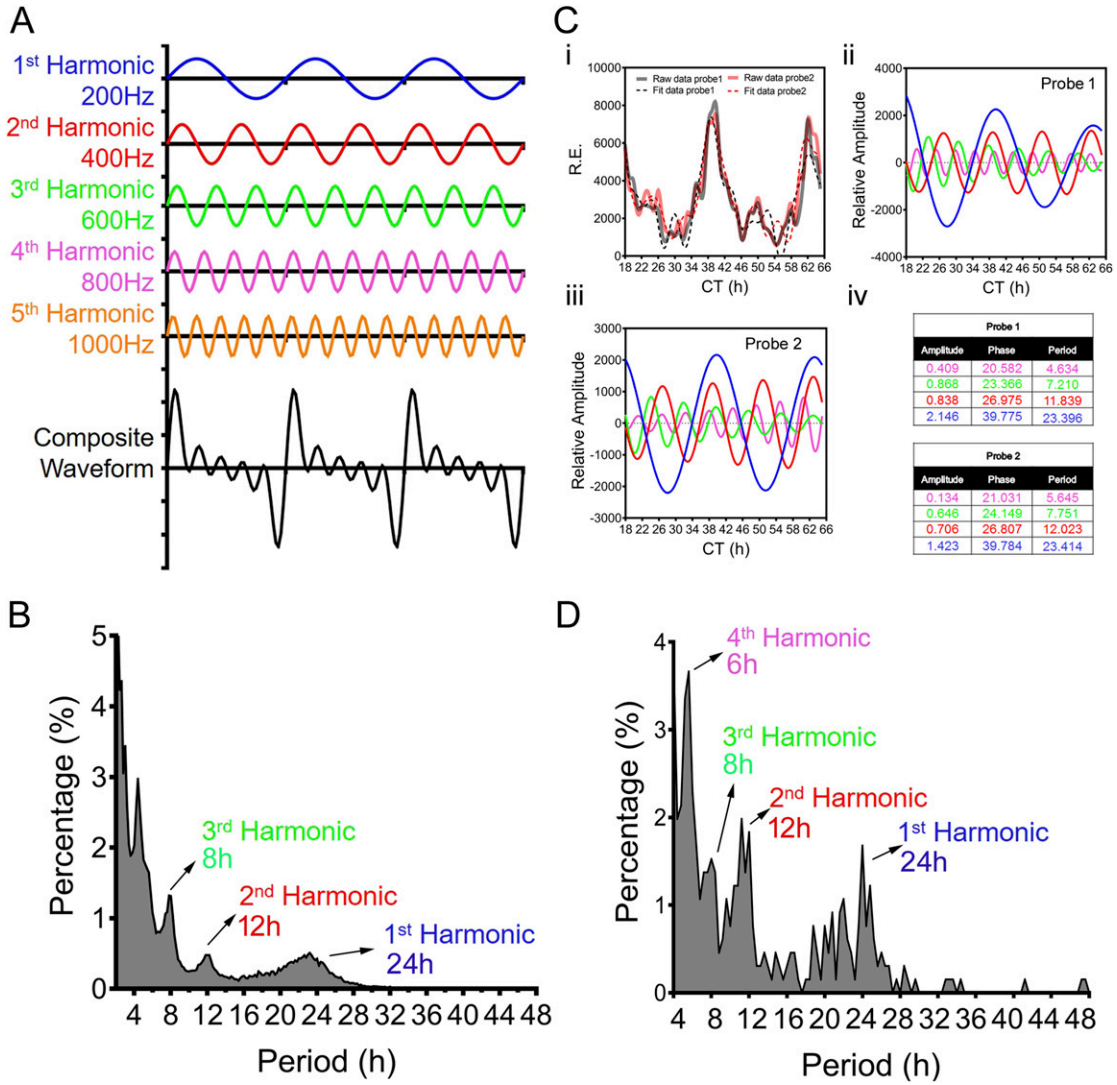


Figure 1. The concept of harmonics in physics and their biological counterpart. (A) Diagram showing the individual harmonics (200 Hz, 400 Hz, 600 Hz, 800 Hz, and 1000 Hz) as well as the composite waveform resulting from adding up all of the harmonics. (B) Distribution of periods of all oscillations identified from the hepatic gene expression microarray dataset [8] via the eigenvalue/pencil approach. The vast majority of oscillations cycle at the first (24-hour), second (12-hour), or third (8-hour) harmonic of the circadian rhythm (24 hours). (C) Representative deconvolution of *Gck* gene mRNA expression by the eigenvalue/pencil method. *Gck* expressions detected by two different probe sets are analyzed by the eigenvalue/pencil method. (i) Raw microarray data (solid line) and model fit (dashed line) for *Gck* hepatic expression as reported in Ref. [8]. Superimposed harmonic oscillations revealed by the eigenvalue/pencil method for probe 1 (ii) and probe 2 (iii) are shown. (iv) Amplitudes, phases, and periods of different oscillations for the two probes with the color matching the different oscillations depicted in (ii) and (iii). (D) Distribution of periods of all oscillations identified from the hepatic metabolites dataset [11] via the eigenvalue/pencil approach. The vast majority of oscillations cycle at the first (24-hour), second (12-hour), third (8-hour), or fourth (6-hour) harmonic of the circadian rhythm (24 hours).

physical oscillations, biological oscillations also are composite rhythms consisting of oscillations of various frequencies [3]. The eigenvalue/pencil method assumes that any oscillation can be approximated by a linear combination of exponentials (sine waves with a decay factor) plus noise and therefore can be applied to any situations where such assumptions are valid. Furthermore, unlike most of the current cycling transcript identification methodologies that

require the user to define a narrow period range [7], the eigenvalue/pencil method does not preassign a period and thus permits the identification of all superimposed oscillations in an unbiased manner [3] (for a more detailed understanding of the strengths and limits of the eigenvalue/pencil method and its wider applications, please refer to Ref. [4]). Oscillations disclosed by this analysis often cycle at the harmonics of the circadian rhythm [3]. For instance, comprehensive analysis of a published mouse liver microarray dataset employing the eigenvalue/pencil method (where liver samples were taken every hour for a total of 48 hours under constant darkness condition) revealed that most oscillations cycle at periods close to 24 hours, 12 hours, 8 hours, and 4 hours, corresponding to the first, second, third, and sixth harmonics of the circadian rhythm (Fig. 1B) [3, 8]. Whereas the ~4-hour oscillations are near the Nyquist limit of sampling frequency and therefore may arise from technical artifacts [9], oscillations cycling with 12-hour and 8-hour periods are thought to be "real" biological oscillations [3]. One example is glucokinase (*Gck*), which encodes for a rate-limiting enzyme involved in both glycogenesis and glycolysis. Whereas *Gck* mRNA expression was originally thought to be solely under the circadian clock control [10], the new analysis revealed that it actually is comprised of superimposed major oscillations cycling at 24-hour, 12-hour, and 8-hour periods with comparable amplitudes (Fig. 1C) [3].

Similar to gene expression oscillations, mammalian metabolic rhythms also are composite oscillations cycling at different harmonics of the circadian rhythm. Analyzing a 1-hour resolution mouse hepatic metabolites profiling dataset [11] revealed that most metabolites consist of superimposed oscillations cycling at 24-hour, 12-hour, 8-hour, and 6-hour periods (Fig. 1D). This is consistent with the observed 24-hour, 12-hour, and 8-hour harmonic oscillations of the respiratory exchange ratio through indirect calorimetry measurement of energy expenditure for mice fed *ad libitum* [3]. Taken together, these unbiased analyses strongly indicate that as proposed thousands of years ago, harmonic resonance is a common "universal" theme that permeates from the physical world all the way to biological systems.

2. The Circatidal Rhythm: The Best Characterized ~12-Hour Cycling Oscillation

The wide prevalence of 12-hour gene expression and metabolic rhythms observed in mouse liver is intriguing. In fact, >20% of all mouse hepatic mRNA and metabolites cycle with an ~12-hour period, of which 4.2% and 11.5%, respectively, are dominant oscillations (~12-hour cycling amplitudes are the largest among all superimposed oscillations) [3]. The prevalent mammalian 12-hour rhythm of gene expression and metabolism is reminiscent of the ~12-hour circatidal rhythms of coastal and estuarine animals that modulate their behavior in tune to the ~12.4-hour ebb and flow of the tides [12]. (For the search related to circatidal rhythms in coastal and estuarine animals, the key words of "circatidal" and/or "tidal rhythms" were used and literatures between 1950 and the present were reviewed; For the 12-hour rhythms in mice, owing to the very limited number of publications on this topic, the authors are very acquainted with the handful of literatures and therefore no search was performed.) Prominent examples include numerous crustaceans such as the fiddler crab (*Uca pugnax*) [13, 14], mudflat crab (*Chiromantes hematocheir*) [15], green shore crab (*Carcinus maenas*) [16, 17], sea louse (*Eurydice pulchra*) [12, 18, 19], *Dimorphostylis asiatica* [20], Chelicerata horseshoe crab (*Limulus polyphemus*) [21], ragworm (*Alitta virens*) [22], Mollusca [23–26], and intertidal insects such as the Asian mangrove cricket (*Apteronemobius asahinai*) [27, 28]. These animals either emerge at low tides to forage, mate, and fight (such as *U. pugnax* and *A. asahinai*) or are more active during high tides when the incoming tide covers their forage and mating grounds (such as *C. maenas* and *E. pulchra*) [12]. More importantly, when removed to the laboratory and divorced from tidal cues, they can still maintain their circatidal locomotive activities at times of expected low water or high water under constant conditions [12–14, 16, 23, 27, 28]. Furthermore, their circatidal rhythms also can be entrained by artificial vibrations or inundation cycles [19, 23, 27]. This free-running behavior combined with entrainability indicates the presence of circatidal clocks in these animals, which under

natural conditions are synchronized to the phase of the tidal cycle encountered on their home environment [12].

So how are these circatidal rhythms generated? Two mechanisms underlying tidal rhythms have been proposed. Some argue that a circatidal clock might be generated by two slightly longer (24.8-hour) circadian clocks acting antiphase to produce 12.4-hour peaks in behaviors such as swimming and foraging [29]. The second school of thought advocates that a dedicated circatidal clock with a period of ~12.4 hours is the basis for circatidal behavior. Superimposed on this tidal clock is a circadian oscillator that drives the day/night modulation observed in locomotor output of crustaceans and crickets [30]. One of the most convincing studies supporting the latter hypothesis was published in *Current Biology* in 2013 [19]. In this elegant study, Zhang *et al.* [19] used two orthogonal approaches (RNA interference against circadian clock genes and bright constant light to disrupt the circadian clock) to abolish the circadian clock in *E. pulchra* and tested their effects on the circatidal swimming rhythms of *E. pulchra*. Although both approaches disrupted the circadian clock as expected, neither influenced the tidal periodicity [19]. Additional studies performed on the American horseshoe crab *Limulus polyphemes* and mangrove cricket (*A. asahinai*) found similar results [30–33]. Collectively, these studies convincingly demonstrate the existence of a dedicated circatidal pacemaker in marine animals that is distinct from the circadian clock and responsible for the establishment of circatidal rhythms.

3. The Prevalence of Mammalian 12-Hour Rhythms

The first study that reported the presence of circadian harmonics of gene expression in mammalian systems, including 12-hour rhythms, was published by John Hogenesch's group [8]. By comprehensively profiling temporal gene expression in mouse liver from animals maintained at constant darkness at high resolution (at 1-hour intervals for a total of 48 hours) using DNA microarray, the authors identified ~200 genes that cycle with a dominant 12-hour period using two statistical methods: a Fisher's G-test and COSOPT [8]. Ingenuity pathway analysis of these 12-hour cycling hepatic genes revealed enriched gene ontology terms, including protein processing and endoplasmic reticulum (ER) homeostasis [8]. Contrary to the evenly distributed phases (the time of peak) of circadian genes, the acrophases of 12-hour cycling hepatic genes are more restricted to the dawn [circadian time (CT)0] and dusk (CT12) of a diurnal cycle, even though the underlying reason was then unknown [8] (By convention, the onset of activity of diurnal organisms defines CT0, whereas the onset of activity of nocturnal organisms defines CT12. Therefore, for nocturnal animals such as mice, they are active from CT12 to CT24). In addition to the liver, they also detected 12-hour cycling transcripts in many different tissues. For example, *Hspa1b*, a heat shock protein 70-kDa family member, showed clear 12-hour transcriptional rhythms in lung, kidney, adrenal gland, heart, and even in the hypothalamus of the central nervous system, indicating that the presence of circadian harmonics is not restricted to the liver [8]. Additionally, in all of these tissues, the phases of peak *Hspa1b* expression are nearly identical, again at dawn and dusk. Since then, a number of studies have confirmed the presence of these 12-hour rhythms of gene expression, especially in mouse liver, using different cycling transcript identification methods [34–37], even though the total repertoire of identified 12-hour cycling transcripts remained small.

We reasoned that the ~200 originally identified 12-hour cycling transcripts were a significant underestimation of the true prevalence of mammalian 12-hour rhythms of gene expression. Because the COSOPT/Fisher's G-test methods used to identify 12-hour cycling transcripts in the original study require the user to preassign a period range, it favors the detection of genes with dominant 12-hour rhythms and is thereby biased against other 12-hour cycling transcripts having superimposed oscillations with larger amplitudes. As expected, when applying the eigenvalue/pencil method on the same mouse microarray dataset, we identified >3000 hepatic genes (~20% of all hepatic transcripts) with 12-hour rhythms [3]. As expected, most of them have superimposed 24-hour circadian rhythms and therefore evade

detection by traditional methods. Among all ~3000 genes, 760 of them are dominant 12-hour cycling genes [3]. Gene ontology analysis of either all or dominant 12-hour cycling genes revealed that, in addition to the expected ER homeostasis and protein quality control pathways, various metabolism-related biological pathways are also highly enriched [3]. Whereas ER-related genes (such as *Eif2ak3*, *Gfpt1*, and *Sec23b*) have dominant 12-hour oscillations and lack superimposed circadian rhythms, 12-hour cycling metabolism genes often have superimposed circadian rhythms (including *Gck*, *Acly*, and *Fasn*) [3].

In addition to the widespread 12-hour cycling mature mRNAs in mouse liver, prevalent 12-hour cycling hepatic protein (~15% of hepatic proteome) also was observed from a time series mass spectrometry dataset [3, 38]. A limited ~35% overlap with 12-hour cycling mRNAs indicates that both transcriptional and posttranscriptional controls contribute to the regulation of 12-hour rhythms of hepatic gene expression [3]. Gene ontology analysis of 12-hour cycling proteins revealed strong enrichment of ER and, intriguingly, mitochondria-associated metabolism pathways, implicating potential coordinated actions of ER and mitochondria in maintaining systemic metabolic homeostasis and stress response [3]. Unlike mature mRNA, the phases of 12-hour protein oscillations are distributed evenly throughout the diurnal cycle, and a clear phase separation of proteins involved in opposing metabolic pathways was found [3].

The prevalent hepatic 12-hour cycling mRNA and proteins involved in metabolic regulation predict a 12-hour rhythm for hepatic metabolites, which is confirmed by *post hoc* analysis of two recently published metabolomics datasets [39, 40]. Eigenvalue/pencil analysis revealed that >20% of hepatic metabolites profiled in these two studies exhibited 12-hour oscillations [3]. Furthermore, joint pathway analysis using MetaboAnalyst [41, 42] showed coordinated 12-hour rhythms of metabolites and gene expressions (Fig. 2A and 2B) [3]. For instance, top-enriched KEGG pathways exhibiting 12-hour rhythms of paired gene expression and metabolites include amino sugar and nucleotide sugar metabolism (*Gfpt1/Gale*: UDP-glucose/UDP-*N*-acetylamino sugars), pyrimidine metabolism (*Uck2*: cytidine/CMP), purine metabolism (*Ppat*/*Gart*/*Atic*/*Adsl*/*Ampd2*/*Nt5e*/*Gmps*: inosine/adenosine/AMP/ADP/ATP/xanthine/guanosine/guanine/GDP), polyamine metabolism (*Srm*/spermidine), glycerophospholipid metabolism (*Pcyt1a*: CDP-choline; *Lpcat3*: 2-Lyso-PC), sphingolipid metabolism (*Sphk2*: sphingosine), pentose phosphate pathway (*Rbks*: ribose), fatty acid biosynthesis (*Fasn*/*Elovl6*: palmitoleate/vaccinate), and amino acid metabolism (*P4ha1*: proline/hydroxyproline), among many others (Fig. 2A and AB) [3].

These highly coupled 12-hour rhythms of hepatic metabolic gene expressions and metabolite oscillations strongly imply the presence of an endogenous 12-hour clock component that is responsible for the precisely timed orchestration of metabolic flux by temporally regulating the expressions of metabolic enzymes. This hypothesis was confirmed experimentally and is discussed in greater detail later. Of particular interest is the prevalent 12-hour hepatic ribonucleoside and ribonucleotide oscillations, which are building blocks for RNA synthesis (Fig. 2C). The acrophases of ribonucleoside and ribonucleotide oscillations are restricted at CT22 and CT34, preceding the two peaks of 12-hour mRNA transcription at CT26 and CT38, implying coordinated purine/pyrimidine metabolism and 12-hour mRNA transcription [3] (Fig. 2C). The 12-hour oscillations of metabolism are not restricted to liver tissues. Indirect calorimetry measurement of real-time mouse energy expenditure revealed the presence of 12-hour harmonics in respiratory exchange ratio oscillations [3], supporting the hypothesis that other peripheral tissues also possess a 12-hour clock that perhaps helps to synchronize 12-hour systemic metabolic homeostasis.

Although the prevalence of 12-hour rhythms of gene expression and metabolism in mice is undisputable, owing to the technical limitations and ethical constraint of obtaining time series tissue samples from humans, the evidence supporting the existence of 12-hour rhythms in human tissues *in vivo* is still largely anecdotal at this time. (For reported ~12-hour rhythms in humans, the key words of "circasemidian" and/or "12-hour rhythm" were searched against literatures published between 1950 and the present.) Nonetheless, in humans, using traditional spectrum analysis methods such as Fourier transform and wavelet analysis,

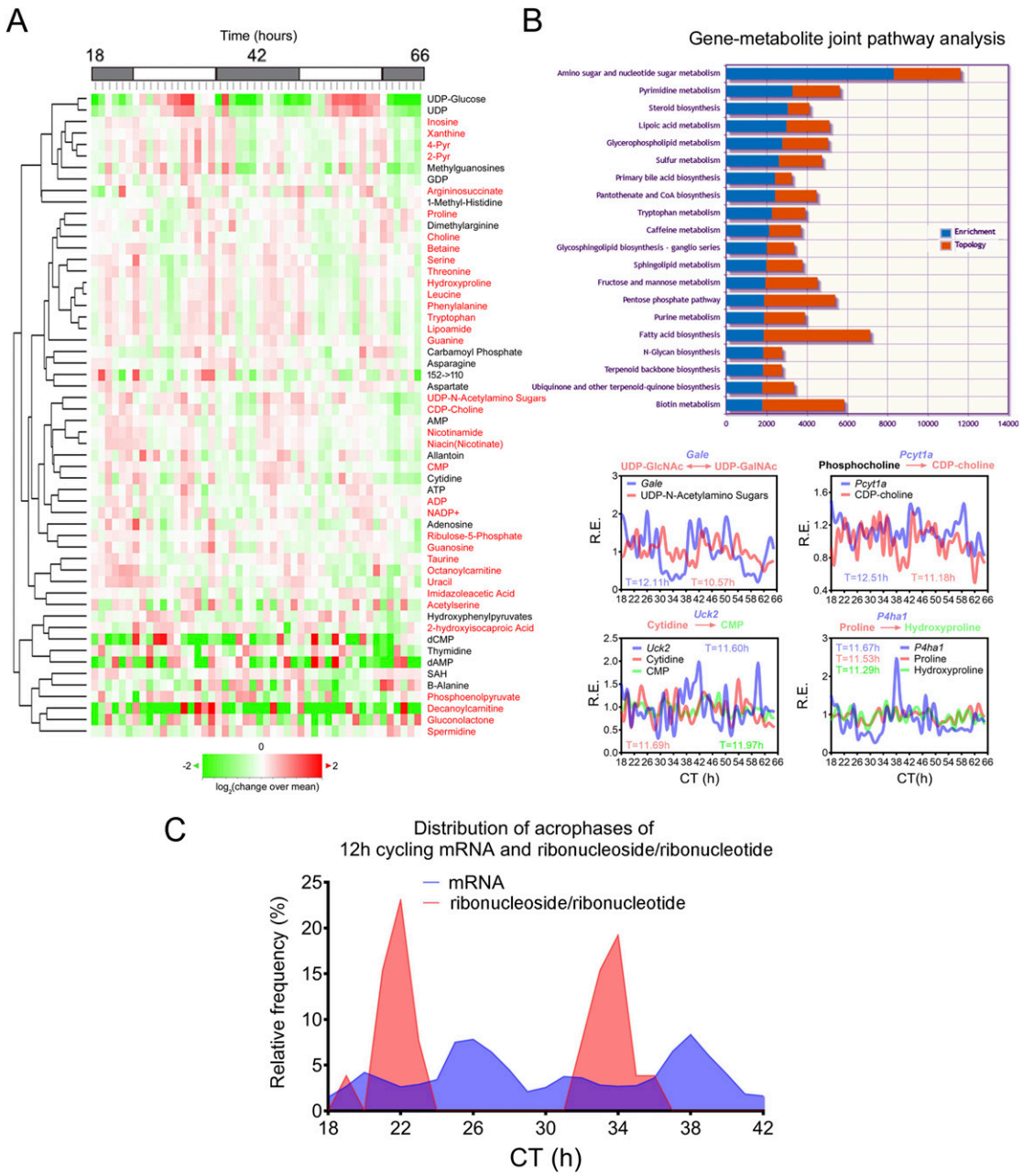


Figure 2. Coupled 12-hour rhythms of hepatic gene expression and metabolism. (A) Heat map of mouse 12-hour cycling hepatic metabolites identified by the eigenvalue/pencil method from published metabolomic dataset [11] after hierarchical clustering. Metabolites with dominant 12-hour oscillations are highlighted in red. (B) (Top) Gene-metabolite joint pathway analysis using MetaboAnalyst [41, 42] reveals top enriched biological pathways. Both enrichment as well as topology scores are shown for each pathway. (Bottom) Representative data showing paired gene expression and metabolite oscillations. (C) Distribution of acrophases of all dominant hepatic 12-hour cycling mRNA and ribonuclesides/ribonucleotides.

rhythms of body temperature [43–48], blood pressure and heart rate variability [49–54], cognitive performance [48, 55–61], migraine onsets and subsequent cerebrospinal fluid sodium level [62], circulating serum and urine metal levels [63], circulating hormone levels [64–66], and sleep patterns [57, 58, 67–74] were all reported to exhibit a 12-hour rhythmic component.

Of these reported 12-hour rhythms in humans, daily variation of temperature and sleep–wake cycles are two of the most interesting. As discussed later, temperature is a strong *Zeitgeber* (a German word for time giver, which refers to a synchronizing agent) for 12-hour rhythms in *C. elegans* [3, 75]. Although it remains unknown whether temperature also can entrain the mammalian 12-hour clock, it is tempting to hypothesize that the 12-hour rhythm of mammalian gene expressions and metabolic rhythms are also in tune to the daily temperature oscillations, similar to what has been observed for the circadian clock [76–78]. As to the 12-hour rhythms of sleep–wake patterns, in addition to the well-characterized peak of sleepiness during the subjective night, a second smaller peak of sleepiness during the early afternoon is a widespread phenomenon (also known as "siesta" in Spanish-speaking countries). Moreover, because the afternoon sleep propensity occurred either under a constant routine [69, 79] or without having lunch [80], it is highly suggested that it is a part of an endogenous biological rhythm. Currently, sleep regulation is conceptualized by the popular "two-process" model that posits that homoeostatic and circadian drives control sleep [81]. The homoeostatic process is controlled by sleep pressure, which accumulates during the course of wakefulness and dissipates during sleep, and is tightly regulated by local changing levels of neuromodulators such as adenosine [82–84]. Alternatively, the sleep–wake cycle during the day and night is regulated by the circadian clock, independent of prior sleep or wake time [81]. Whereas it has long been thought that the afternoon sleepiness is under the homeostatic sleep control, several studies challenged this notion and suggested that the afternoon sleep propensity may instead reflect an endogenous 12-hour cycle of slow wave sleep (which is also referred to as nonrapid eye movement sleep or in more colloquial terms "deep sleep"), independent of duration of prior wakefulness [61, 69, 74]. Although it has been convincingly demonstrated that slow wave sleep can be strongly induced by local increased levels of adenosine in the basal forebrain through its adenosine A1 receptor and repressed by its antagonist caffeine [82, 85], the mechanism of how the concentration of adenosine is regulated in the brain remains elusive. Intriguingly, adenosine and three of its main precursors (ATP, AMP, and SAH) all exhibit robust 12-hour rhythms in mouse liver (Fig. 2A and 2B). Furthermore, xanthine, a close relative of caffeine (which is a methylxanthine) also revealed a 12-hour rhythm (Fig. 2A). Although it is premature to conclude that local intracellular and extracellular levels of adenosine and xanthine derivatives may also exhibit 12-hour rhythms in the central nervous system and is coupled to the 12-hour rhythms of slow wave activity cycle, these intriguing observations and correlations warrant future studies to either prove or refute this hypothesis.

4. Evidence Supporting the Existence of a Cell-Autonomous Mammalian 12-Hour Clock

Two pertinent questions arise regarding the mammalian 12-hour rhythms. First, are the 12-hour oscillations of gene expression and metabolism cell-autonomous? Second, are 12-hour rhythms established by a dedicated mammalian 12-hour clock or are they regulated by some variants of the well-characterized circadian clock? Theoretically, at least three possibilities exist. In the first scenario, the 12-hour rhythms are not cell-autonomous in nature. It is possible that one of the two peaks is established by the circadian clock and the second peak results from physiology-associated systemic cues only found *in vivo*. In the second scenario, the 12-hour rhythm is cell-autonomous. However, it is established by two circadian transcription activators or repressors appearing in antiphase and therefore is dependent on the circadian clock [36]. In the third scenario, the mammalian 12-hour rhythms are not only cell-autonomous, but also are established by a dedicated 12-hour clock "separate" from the circadian clock, a mechanism similar to the independent circatidal clock in certain crustaceans and intertidal insects [19, 32, 33].

Early studies favor the first hypothesis (the 12-hour rhythms are not cell-autonomous and are established by the combined effects of circadian clock and fasting–feeding cues) due to the following three major observations. (1) No statistically significant 12-hour rhythms of gene

expression were detected in forskolin-synchronized NIH3T3 cells, implying that mammalian 12-hour rhythms are not cell-autonomous (Fig. 3B) [8]. (2) Eight-hour daytime-restricted feeding substantially impairs the 12-hour rhythms of several hepatic gene expressions. These genes still maintained peak expression at approximately CT26, coinciding with the new feeding time; however, the subjective evening peak was largely absent. This evidence suggests that at least one component of the 12-hour rhythm is driven by feeding [8]. (3) Brain-specific rescue of *Clock*$^{\Delta 19}$ mutant mice converted hepatic 12-hour rhythms into 24-hour rhythms, suggesting that signaling via the central circadian oscillator may be required to generate one of the two daily peaks of expression [37]. Whereas these results could suggest the lack of a cell-autonomous mammalian 12-hour clock, in light of strong new evidence supporting the existence of a cell-autonomous mammalian 12-hour clock [3], alternative interpretations of these data are offered and will be elaborated on in greater detail below.

An initial clue hinting at the possible existence of an independent mammalian 12-hour clock comes from the observed mathematical orthogonal relationship between superimposed 12-hour and circadian oscillations uncovered for most of the 12-hour cycling ER homeostasis and metabolism genes [3]. Subsequent *post hoc* analysis of hepatic RNA sequencing data in wild-type and whole-body *BMAL1* knockout mice fed *ad libitum* (both conventional and adult *BMAL1* deletion mice [86]) confirmed the independence of 12-hour rhythms of key ER and

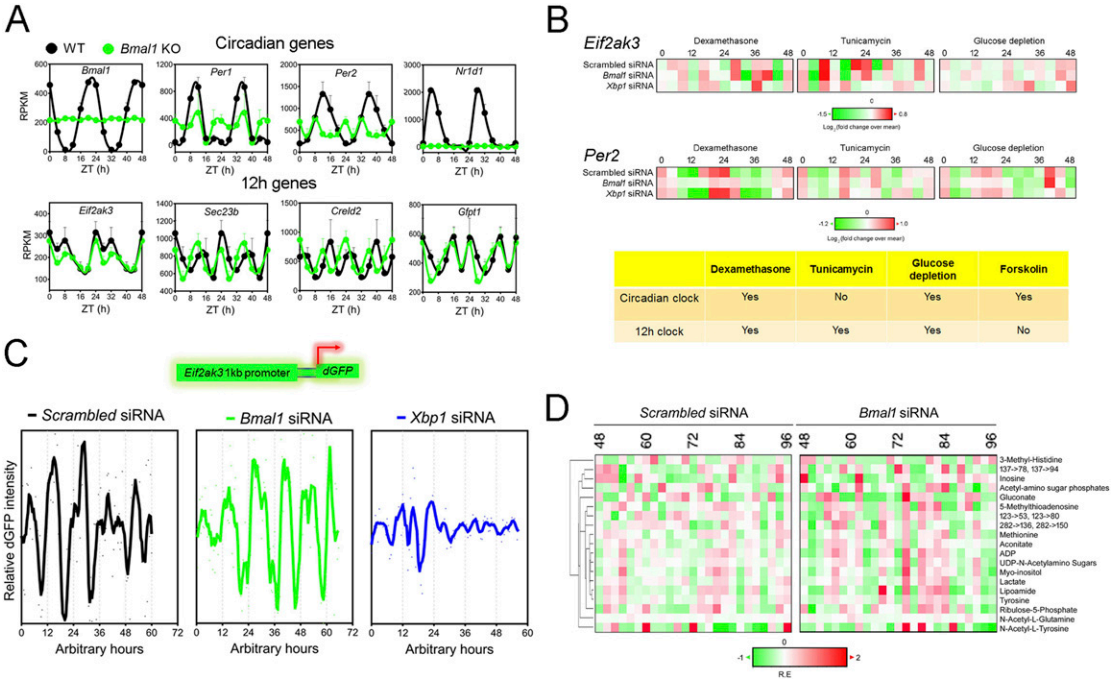


Figure 3. Twelve-hour rhythms of gene expression and metabolism are cell-autonomous, established by a dedicated 12-hour clock and evolutionarily conserved. (A) Representative reads per kilobase of transcript per million mapped reads (RPKM) of normalized hepatic circadian (top) as well as 12-hour cycling (bottom) gene expression from wild-type (WT) and conventional *BMAL1* knockout (KO) mice under 12-hour/12-hour light/dark conditions as reported in Ref. [86]. Data are graphed as the mean ± SEM (n = 4) and double plotted for better visualization. (B) Heat map representation of oscillations of *Eif2ak3* and *Per2* mRNA level after dexamethasone, tunicamycin, or glucose depletion shock treatment in MEFs. The heat map is derived from quantitative PCR data reported in Ref. [3]. A summary of the conclusion is shown in the table below. (C) Representative recordings of single-cell time lapse microscopy analysis of *Eif2ak3* promoter-driven dGFP oscillation in scrambled siRNA, *Bmal1* siRNA, or *Xbp1* siRNA transfected MEFs. (D) Heat map of 12-hour cycling metabolites in dexamethasone-synchronized human U2OS cells under both *scrambled* siRNA and *Bmal1* siRNA transfection conditions compiled from Ref. [11]. Twelve-hour cycling metabolites are identified by the eigenvalue/pencil method [3].

metabolism gene expressions from the circadian clock [3]. Specifically, for 12-hour cycling genes lacking superimposed circadian rhythms (such as *Eif2ak3*, *Gfpt1*, *Creld2*, and *Sec23b*), their 12-hour rhythms are almost identical between wild-type and *BMAL1* knockout mice under either a 12-hour/12-hour light/dark schedule or a constant darkness condition [3, 86] (Fig. 3A). For 12-hour cycling genes with superimposed circadian rhythms (such as *Gck*, *Fasn*, *Dnajb4*, and *Chka*), more perceptible 12-hour rhythms were observed in *BMAL1* knockout mice [3, 86]. The independent relationship between 12-hour and 24-hour rhythms for these ER homeostasis and metabolism genes also was verified in $Clock^{\Delta 19}$ mutant mice [3, 87].

Can the 12-hour rhythm of gene expression be found *in vitro* or does its establishment require systemic cues only found *in vivo* as previously suggested [8, 35]? In light of the newly found independence of 12-hour cycling genes from the circadian clock, an alternative interpretation of the lack of observed 12-hour rhythms in forskolin-synchronzied NIH3T3 cells [8] is that forskolin is capable only of synchronizing the circadian clock but not the circadian clock-independent 12-hour clock. Consistent with this hypothesis, it was found that the circadian and 12-hour cycling genes are responding to a different repertoire of external cues (Fig. 3B) [3]. Whereas the cAMP inducer forskolin is only a strong synchronizer of the mammalian circadian clock [8], the ER stress inducer tunicamycin (which blocks UDP-GlcNAc–mediated UDP-*N*–linked glycosylation) can only synchronize the 12-hour clock [3] (Fig. 3B). This is consistent with observed dominant circadian and 12-hour rhythms of cAMP and UDP-*N*-acetylamino sugars levels, respectively, in mouse liver [11] (Fig. 2B). Contrary to the discriminatory nature of forskolin and tunicamycin, dexamethasone and glucose depletion can synchronize both the circadian and 12-hour clocks [3] (Fig. 3B). More importantly, whereas siRNA-mediated depletion of *Bmal1* abolishes dexamethasone and glucose depletion–synchronized circadian *Per2* expression, it does not affect dexamethasone, glucose depletion, or tunicamycin-synchronized 12-hour rhythms of *Eif2ak3* expression in mouse embryonic fibroblasts (MEFs) [3] (Fig. 3B). Moreover, by cloning the *Eif2ak3* promoter before a destabilized green fluorescence protein (dGFP) and performing time-lapse imaging of dGFP intensity in single cells without any prior perturbation, robust 12-hour oscillations were observed in MEFs [3], which also was not affected by *Bmal1* siRNA knockdown (Fig. 3C) [3]. Lastly, 37 metabolites of the 137 examined also exhibited ~12-hour rhythms in dexamethasone-entrained human U2OS cells and 19 of them still maintained ~12-hour rhythms in the presence of *Bmal1* knockdown [11] (Fig. 3D). Taken together, these observations strongly support the hypothesis that the 12-hour rhythm of gene expression, especially those involved in ER/metabolic pathways, and subsequent 12-hour rhythms of metabolism, are cell-autonomous and driven by a dedicated 12-hour pacemaker "distinct" from the circadian clock in mammalian cells [3].

Although the 12-hour rhythms of gene expression and metabolism are cell-autonomous, they can be disrupted by altered fasting–feeding cycles. Daytime-restricted feeding negatively influences 12-hour rhythms of hepatic gene expression [8]. Consistent with this finding, daytime-restricted feeding also substantially impairs 12-hour oscillations of respiratory exchange ratio in the mouse [3]. Thus, one also can envision how the circadian and 12-hour clocks may interact indirectly. For instance, manipulation/perturbation of the central circadian clock in the brain can lead to altered feeding–fasting and wake–sleep behaviors, which in turn may feed back to the 12-hour clock to alter 12-hour rhythms of gene expression and metabolism in peripheral tissues. In fact, this may very well account for the observed phase shift in several 12-hour cycling hepatic genes as well as the varied 12-hour cycling transcriptome reported in different circadian deficient mice models [3, 35, 86, 87]. Additionally, because many of the 12-hour cycling genes have superimposed circadian rhythms, alteration of the circadian clock can have a major influence on the overall gene oscillations, without necessarily affecting the superimposed 12-hour rhythms. This could be the case for the observed conversion of certain hepatic 12-hour rhythms into 24-hour rhythms upon brain-specific rescue of $Clock^{\Delta 19}$ mutant mice [37].

Whereas these new data support the existence of a cell-autonomous mammalian 12-hour clock, they do not rule out the possibility that certain 12-hour rhythms are influenced by the

circadian clock and/or the effects of certain external cues and are not cell-autonomous. Future studies are needed to profile the complete 12-hour transcriptome under the 12-hour clock control, as well as to investigate the physiologic conditions by which these distinct clocks can interact systemically in multiple model organisms.

5. The Spliced Form of XBP1 Transcriptionally Regulates the 12-Hour Clock

How is the mammalian 12-hour clock regulated? Observed prevalent 12-hour rhythms of hepatic nascent mRNA and enhancer RNA expression indicate that the mammalian 12-hour clock is regulated primarily at the transcriptional level [3, 88]. Consistent with the strong enrichment of ER stress and unfolded protein response (URP) pathways in 12-hour cycling hepatic transcriptome (for a review of mammalian UPR, see Refs. [89–91]), 12-hour cycling of UPR transcription factor spliced XBP1 (XBP1s) and ATF4 was found in mouse liver under both a 12-hour/12-hour light/dark schedule and constant darkness conditions with peak expressions found at CT10 to CT12 and CT22 to CT24, consistent with the acrophases of 12-hour cycling hepatic transcriptome [3, 35]. Furthermore, 12-hour cycling of XBP1s expression was found to be induced in tunicamycin-synchronized MEFs and is independent from the circadian clock [3]. Intriguingly, both the total level of *Xbp1* mRNA (the combined level of *Xbp1*s and *Xbp1*us) and its splicing efficiency exhibited robust 12-hour rhythms both in mouse liver *in vivo* and in MEFs *in vitro* [3, 35]. The 12-hour rhythms of *Xbp1* splicing efficiency and *XBP1s* expression *in vivo* is further supported by the observed 12-hour rhythms of its upstream endoribonuclease IRE1α phosphorylation at Ser724 [35].

Twelve-hour rhythms of nuclear XBP1s levels correlate with the 12-hour rhythms of chromatin recruitment of XBP1s to the promoters of several 12-hour cycling genes both in mouse liver *in vivo* and in tunicamycin-synchronized MEFs *in vitro* [3]. Knockdown of *Xbp1* inhibits both tunicamycin and glucose depletion–synchronized 12-hour rhythms of ER homeostasis (such as *Eif2ak3*, *Ddit3*, *Sec23b*, and *Herpud1*) and metabolism (such as *Gfpt1* and *Acly*) gene expression in MEFs [3] (Fig. 3B). Additionally, knockdown of *Xbp1* abolished cell-autonomous 12-hour rhythms of *Eif2ak3* promoter–driven dGFP intensity in a single MEF cell (Fig. 3C). In contrast, ablation of *Xbp1* has no effects on dexamethasone and glucose depletion–synchronized circadian gene expression [3] (Fig. 3B). The transcriptional regulation of mammalian 12-hour transcriptome by XBP1s is further consistent with numerous past reports on the transcriptional regulation of the same repertoire of genes by XBP1s during pathological conditions of ER stress [92–95]. This evidence suggests that similar to the circadian clock, the mammalian 12-hour clock is also subject to major transcriptional control. Note that so far, the evidence supporting the role of *Xbp1*s in the regulation of the mammalian 12-hour clock is solely restricted to the study of a few 12-hour rhythmic genes in MEFs *in vitro*, and future studies using XBP1s knockout mice models are needed to comprehensively profile the XBP1s-regulated 12-hour transcriptome in an unbiased manner.

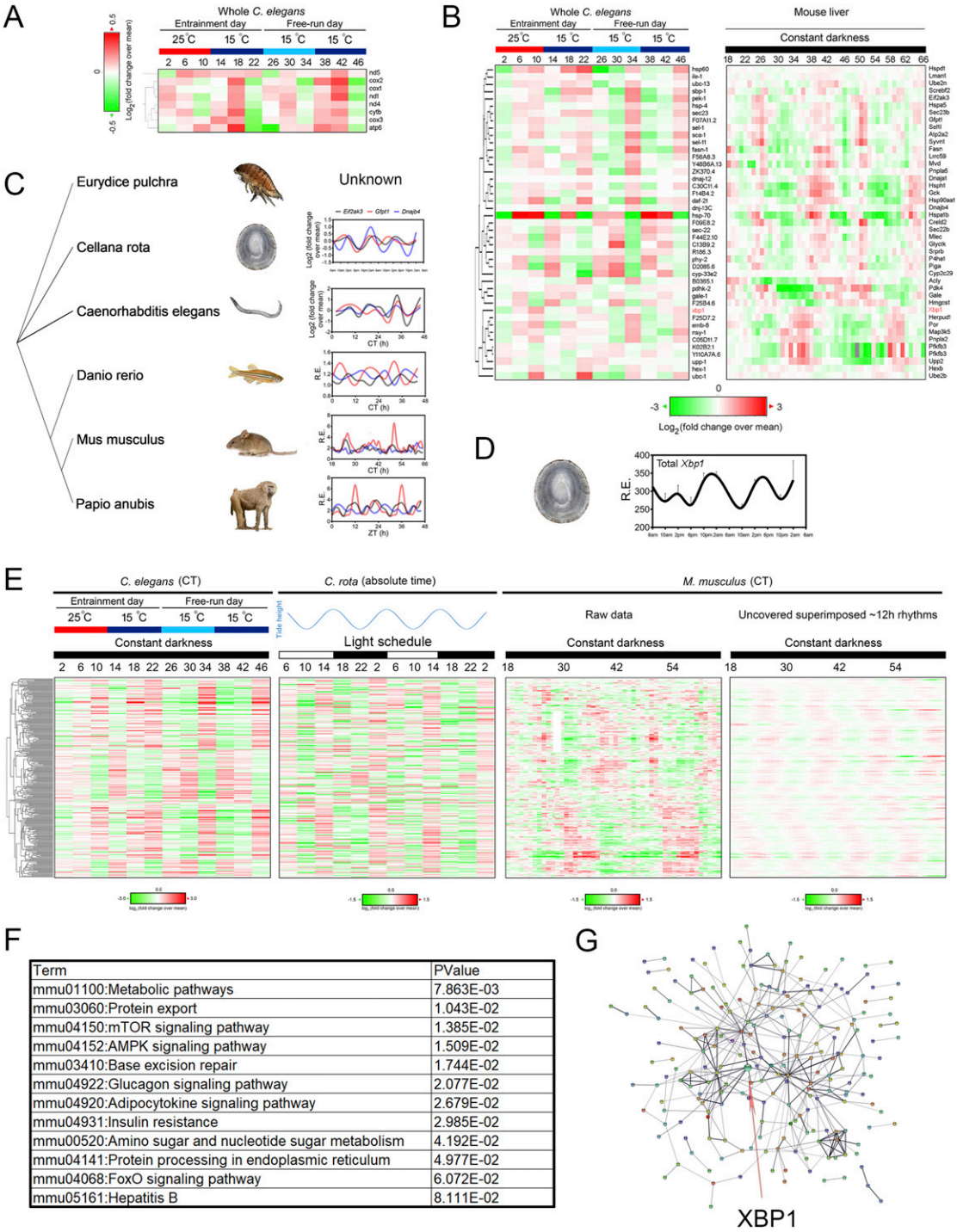
6. The Mammalian 12-Hour Clock Is Evolutionarily Conserved and May Be Circatidal in Origin

From where does the mammalian 12-hour clock originate? Given the distinctions of the circadian clock from the 12-hour clock/circatidal clock in both mouse and *E. pulchra* [3, 19] and the fact that mammals share common marine ancestors, it is logical to postulate that the mammalian 12-hour clock evolved from the ancient circatidal clock. Previous work revealed robust 12-hour circatidal mRNA rhythms for 10 mitochondrial DNA (mtDNA)–encoded protein-coding genes (*Mt-Nd*1~6, *Mt-Cox*1~3, and *Cytb*) that encode mitochondrial components of complexes I (NADH dehydrogenase) and IV (cytochrome *c* oxidase) in *E. pulchra* under free-running conditions, which are correlated with circatidal rhythms of oxygen consumption in the same animals [18, 96]. *Post hoc* analysis of two published mouse hepatic Gro-Seq [88] and Nascent-Seq [97] datasets that measure nascent RNA transcription rate

revealed, surprisingly, conserved 12-hour rhythms of mtDNA-encoded gene transcription [3] These 12-hour rhythms of mtDNA-encoded gene expression were further confirmed at mature mRNA and protein levels in mouse liver [3, 38, 40]. Furthermore, cell-autonomous 12-hour rhythms of mtDNA-encoded gene expression were found in glucose depletion–synchronized MEFs in a BMAL1-independent manner [3]. Also supporting the hypothesis that the mammalian 12-hour clock evolves from a circatidal origin is the observed similar free-running periodicity of *Eif2ak3* promoter–driven dGFP oscillation in MEFs (12.6 hours) with that of circatidal activity rhythms reported in *E. pulchra* after tidal cue entrainment (12.7 hours) and in the mangrove cricket, *A. asahinai* (12.6 hours to 12.9 hours) [19, 32, 33].

In addition to crustaceans and mammals, strikingly, mtDNA-encoded gene transcription was found in 12-hour/12-hour warm/cold temperature cycles-entrained as well as free-running *C. elegans* [75] (Fig. 4A). In fact, most of the *C. elegans* homologs of core 12-hour cycling mammalian transcriptome involved in ER homeostasis and metabolism revealed robust 12-hour rhythms under the same conditions [3, 75] (Fig. 4B). These include the *C. elegans* homologs of dominant 12-hour cycling mammalian genes such as total *Xbp1*, *Eif2ak3*, *Hspa1b*, *Gfpt1*, *Hspa5*, *Sec23b*, *Creld2*, *Dnaja1*, and *P4ha1*, with *Hspa1b/hsp-70* being the strongest oscillating gene in both species (Fig. 4B). It is important that for mammalian 12-hour cycling genes with superimposed dominant circadian rhythms (such as *Pfkfb3*, *Por*, *Map3k5*, and *Upp2*), 12-hour oscillations are the dominant ones in *C. elegans* (Fig. 4B). This could be explained by the absence of oscillations of the canonical circadian clock genes in *C. elegans* [3, 75, 98]. Further to this point, these 12-hour rhythms oscillate more weakly or are completely absent in free-running *C. elegans* that are previously entrained by the 12-hour/12-hour light/dark cycles [75]. Taken together, this evidence indicates that the *C. elegans* 12-hour clock is responding to temperature, rather than light, cues, which is justified by the overall lack of exposure to light in their natural soil habitat. In addition to the nematode, 12-hour oscillations of ER homeostasis and metabolism gene expression also were found in light-entrained and free-running zebrafish [99] as well as in the liver of light-entrained baboons [100] (Fig. 4C). Most intriguingly, by thoroughly analyzing published time series database, we further identified a total of 280 genes (with a P value of 1.30×10^{-187} for observing the same number of conserved 12-hour genes by chance) that exhibited conserved ~12-hour rhythms in temperature-entrained *C. elegans* [75], naturally caught *Cellana rota* (a mollusc captured from its natural intertidal habitat and exhibiting a dominant circatidal clock) [23] and mouse liver [8] (Fig. 4E). As expected, gene ontology analysis of these 280 common ~12-hour cycling genes reveals enriched biological pathways in metabolic control and ER homeostasis (Fig. 4F).

Although it is much appreciated that the molecular mechanisms for generating the circadian clock, namely, the transcriptional/translational feedback loop (TTFL), are conserved in multiple species, there is little overlap in the individual genes involved in the establishment of the circadian TTFL clockwork in different species. Therefore, many thought that the circadian clock evolved separately in different lineages [101]. However, the fact that the core 12-hour clock genes remained conserved even in divergent species that cross phylum boundaries (Fig. 4C) strongly implies that the 12-hour clock may even be more ancient and evolved earlier than the circadian clock. Further supporting this hypothesis is the conserved 12-hour oscillation of the proposed transcriptional regulator of the mammalian 12-hour clock, *Xbp1* in *C. rota*, whose oscillation is also in phase with the general circatidal transcriptome peaking at rising tides [23] (Fig. 4D and 4E). Additionally, computational construction predicted a mammalian interactive network using these 280 genes via STRING [102] and put XBP1 in the center of the network connecting different peripheral subhubs (Fig. 4G), consistent with the experimental results demonstrating the essential role of *Xbp1* in regulating the 12-hour clock in MEFs (Fig. 3). Notwithstanding the evidence, the causal relationships between the circatidal *Xbp1*/UPR-related gene oscillations and the circatidal behavior in these marine animals remain to be determined.



Term	PValue
mmu01100:Metabolic pathways	7.863E-03
mmu03060:Protein export	1.043E-02
mmu04150:mTOR signaling pathway	1.385E-02
mmu04152:AMPK signaling pathway	1.509E-02
mmu03410:Base excision repair	1.744E-02
mmu04922:Glucagon signaling pathway	2.077E-02
mmu04920:Adipocytokine signaling pathway	2.679E-02
mmu04931:Insulin resistance	2.985E-02
mmu00520:Amino sugar and nucleotide sugar metabolism	4.192E-02
mmu04141:Protein processing in endoplasmic reticulum	4.977E-02
mmu04068:FoxO signaling pathway	6.072E-02
mmu05161:Hepatitis B	8.111E-02

Figure 4. Twelve-hour rhythms of gene expression are evolutionarily conserved. (A) Heat map of temporal mtDNA-encoded gene expression in temperature-entrained as well as free-running *C. elegans* compiled from Ref. [75]. (B) Heat map of core ER homeostasis and metabolism related 12-hour cycling gene expression in both temperature-entrained as well as free-running *C. elegans* (left; compiled from Ref. [75]) and mouse liver under constant darkness (right; compiled from Ref. [8]). *Xbp1* is highlighted in red. (C) Phylogenetic tree and relative mRNA expression of *Eif2ak3*, *Gfpt1*, and *Dnajb4* in *C. rota* (second row; reported in Ref. [23]), *Caenorhabditis elegans* (third row; reported in Ref. [75]), *Danio rerio* (fourth row; reported in Ref. [99]), *Mus musculus* (fifth row; and reported in Ref. [8]), and *Papio anubis* (last row; reported in Ref. [100]) during a 48-hour interval. The status of the three genes was not reported for *E. pulchra* in the study [18]. The data for *Papio anubis* is double plotted for

better visualization. (D) The mRNA level of *Xbp1* ortholog in *C. rota* captured from different times of day from their natural intertidal habitat in the wild as compiled from Ref. [23]. (E) Heat map of all 280 evolutionarily 12-hour cycling gene expression in temperature-entrained as well as free-running *C. elegans* (left; compiled from Ref. [75]), naturally caught *C. rota* in tune to 12-hour cycling tidal cues under 12-hour/12-hour natural light condition (middle; compiled from Ref. [23]), and mouse liver under constant darkness (right; compiled from Ref. [8]). (F) Gene ontology analysis of enriched KEGG pathways from these 280 genes. (G) Predicted interactive network construction of these 280 proteins using STRING [102] with XBP1 highlighted by the arrow.

7. The Functions of the Mammalian 12-Hour Clock

It has been widely accepted that all organisms adjust their biochemical, physiological, and behavioral processes in the most advantageous manner, in phase with predictable environmental changes [103]. Alignment of endogenous clocks with environmental timing increases an organism's overall fitness and survival, whereas misalignment between the two features considerably reduces an organism's fitness and can be a strong predisposing factor to multiple morbidities in almost all species examined [104–111]. Whereas this conclusion was based mostly on studies on the circadian clock, it is no exception for the 12-hour clock. Intertidal animals exhibiting dominant circatidal clocks tune their tidal rhythmicity to ~12-hour rhythmic cycles of inundation and exposure that give rise to rapid changes in temperature, salinity, hydrostatic pressure, osmotic pressure, food, and predation pressure [112].

The reasons for the existence of a 12-hour clock in mammals are, however, subtler. After all, it has been a long time since we "came out of the sea," and mammals no longer experience 12-hour environmental changes of salinity, temperature, or hydrostatic pressure. Instead, we are constantly exposed to circadian changes of temperature, light, and food availability and, as a result, the circadian clock has long been thought to be the only clock present in higher organisms, including mammals. Nonetheless, if the theory that biological clocks are always in synchronization with environmental timing also holds true for the mammalian 12-hour clock, then we must have either overlooked some 12-hour cycling environmental factors to which the mammals are exposed to everyday, or there is "something else" cycling with a 12-hour period that is indirectly caused by the circadian environmental changes of temperature, light, or food commonly associated with land mammals.

We favor the latter hypothesis and conjecture that the mammalian 12-hour clock is in tune to a 12-hour cycling "stress cycle" or, more specifically, a 12-hour cycling "metabolic stress cycle" that results from the innate misalignment between energy intake and energy expenditure within a 24-hour window (Fig. 5A). The early clues suggesting the existence of such a 12-hour cycling metabolic cycle are the unique dawn and dusk phase distribution of 12-hour cycling mRNAs in mouse liver, both of which correspond to transition periods between fasting–feeding and sleep–wake (Fig. 2C) [3]. At the subjective dawn [Zeitgeber time (ZT)10 to ZT12 for the nocturnal mouse, where ZT0 is the time of light on and ZT12 is the time of light off), the prolonged absence of energy intake from the subjective night combined with reduced but still notable energy expenditure (during sleep the brain and other parts of the body still consume large amounts of energy to consolidate memory, dispose of the metabolic waste, and repair the body [113–115]) leads to a peak of energy "overdraft." In contrast, at the subjective dusk (ZT0 to ZT2 for nocturnal mice), sufficient energy intake during the subjective day plus substantially reduced energy expenditure gives rise to a peak of energy "excess" (Fig. 5A). Both energy overdraft and energy excess create great metabolic stress for the mammalian cells. They activate the same stress response pathways to mitigate the original stress, including the two most well-known stress responses that evolved to cope with such metabolic stress: the classical ER-associated unfolded protein response (UPR^{ER}) [90] and a second stress response pathway reacting to unfolded proteins in the mitochondria coined "mitochondrial UPR" (UPR^{mt}) [116–119].

For example, both fasting-associated hypoglycemia and feeding-associated hyperglycemia conditions can activate the UPR^{ER} [120–123]. Likewise, both increased and decreased

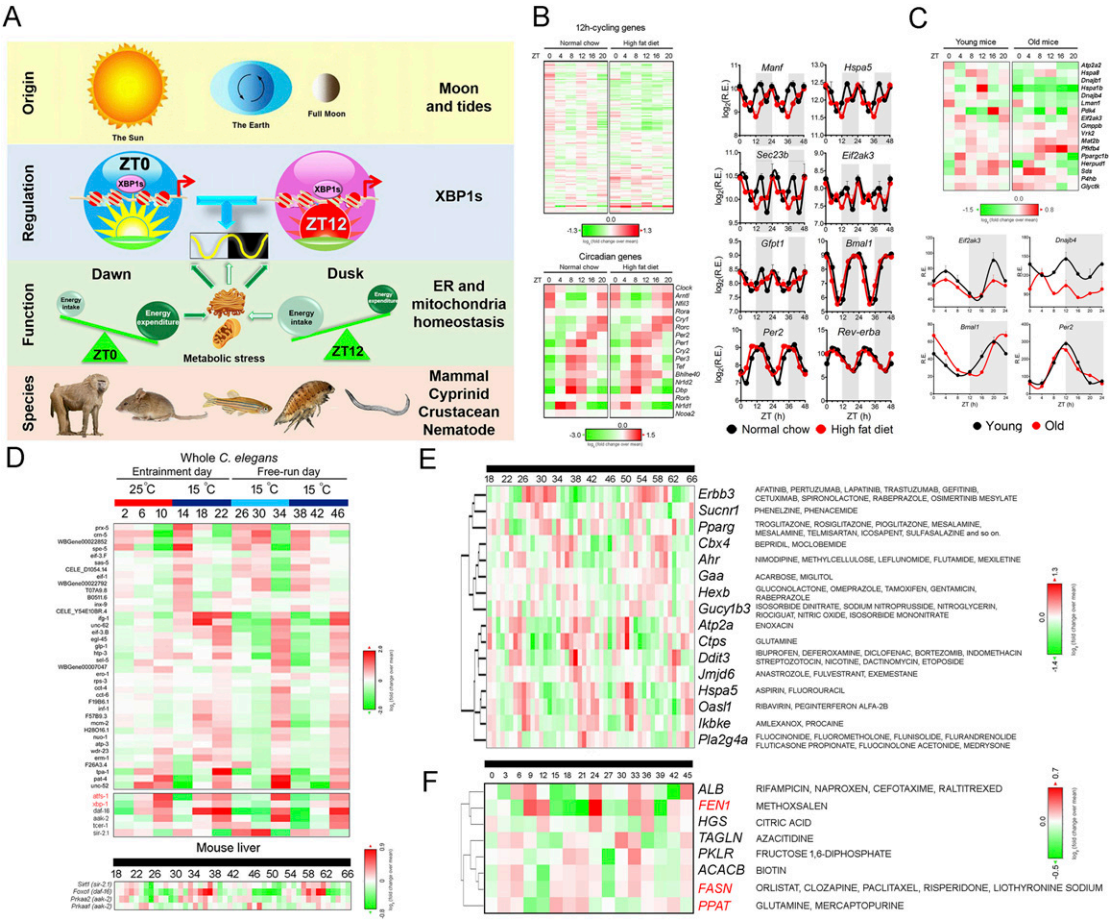


Figure 5. Mammalian 12-hour clock in diseases and chronotherapy. (A) Diagram summarizing the origin, regulation, function, and species conservation of 12-hour clock. Please see the main text for detailed description of each section. (B) High-fat diet disrupts the hepatic 12-hour cycling, but not circadian gene expression in mice. Heat map (left) and representative $\log_2$ normalized expression (right) of key 12-hour cycling and circadian genes under normal chow and high-fat diet conditions are compiled from Ref. [40]. The data are double plotted for better visualization. (C) Disrupted hepatic 12-hour rhythms are associated with aging. Heat map (top) and representative mRNA expression (bottom) of key 12-hour cycling and circadian gene in young and old male mice are compiled from Ref. [152]. (D) Aging-regulating genes are enriched for 12-hour rhythmicity in *C. elegans*. (Top) Heat map of aging-related 12-hour cycling gene expression in both temperature-entrained as well as free-running *C. elegans* as compiled from Ref. [75]. Of 62 genes that increase worm lifespan by 10% when knocked down postdevelopmentally [163], 38 (62%) of them showed 12-hour rhythm in temperature-entrained and free-running worms as shown in the heat map. Additionally, positive regulators of aging, including *atfs-1*, *xbp-1*, *daf-16*, *aak-2*, *tcer-1*, and *sir-2.1*, all exhibited 12-hour rhythms of gene expression. (Bottom) Heat map of mammalian ortholog of *sir-2.1* (*Sirt1*), *daf-16* (*Foxo1*), and aak-2 (*Prkaa1* and *Prkaa2*) expression in mouse liver under constant darkness condition as compiled from Ref. [8]. *Atfs1* and *Xbp1* are highlighted in red. (E and F) Twelve-hour clock–based chronotherapy blueprint. Heat map of temporal mRNA (E) and protein (F) expression of hepatic 12-hour cycling genes with Food and Drug Administration–approved drug interactions as compiled from Refs. [8, 38]. The names of drug are indicated on the right. *FEN1*, *FASN*, and *PPAT* also exhibit 12-hour rhythms of mRNA expression and are highlighted in red.

mitochondria respiration capacity can activate UPR^{mt} and other mitochondrial stress pathways [124]. Therefore, it is not surprising that both UPR^{ER} transcription factor *xbp-1* and UPR^{mt} transcription factor *atfs-1* exhibit robust 12-hour rhythms of expression peaking at dawn (C10) and dusk (CT22), synchronized to the 12-hour rhythms of proposed cellular

metabolic stress (Fig. 5A and 5D). Whereas the true mammalian homolog of *atfs-1* remains elusive at this time, one potential candidate *Ddit3/Chop* [117] does exhibit a robust 12-hour rhythm in mice [3]. Based on these findings, we propose that the mammalian 12-hour clock may govern physiological 12-hour oscillations of UPR^{ER} and UPR^{mt} to maintain metabolic homeostasis. These oscillations are further synchronized to the energy imbalance–induced 12-hour metabolic stress cycle occurring in the ER and mitochondria [3]. Therefore, we coined the term CREMA (coordinated rhythms of ER and mitochondria action) to reflect the coupled 12-hour rhythms of gene expression involved in both ER and mitochondria homeostasis [3]. Supporting the existence of such a 12-hour metabolic stress cycle are observed 12-hour cycling metabolites (such as ADP and inosine) in dexamethasone-synchronized human U2OS cells with or without *BMAL1* knockdown (Fig. 3D) [11]. Nevertheless, more studies utilizing real-time imaging of stress-related metabolites in single cells are needed to substantiate that such a 12-hour metabolic stress cycle indeed exists in a fully cell-autonomous manner as predicted.

8. The Potential Roles of the 12-Hour Clock in Diseases and Therapy

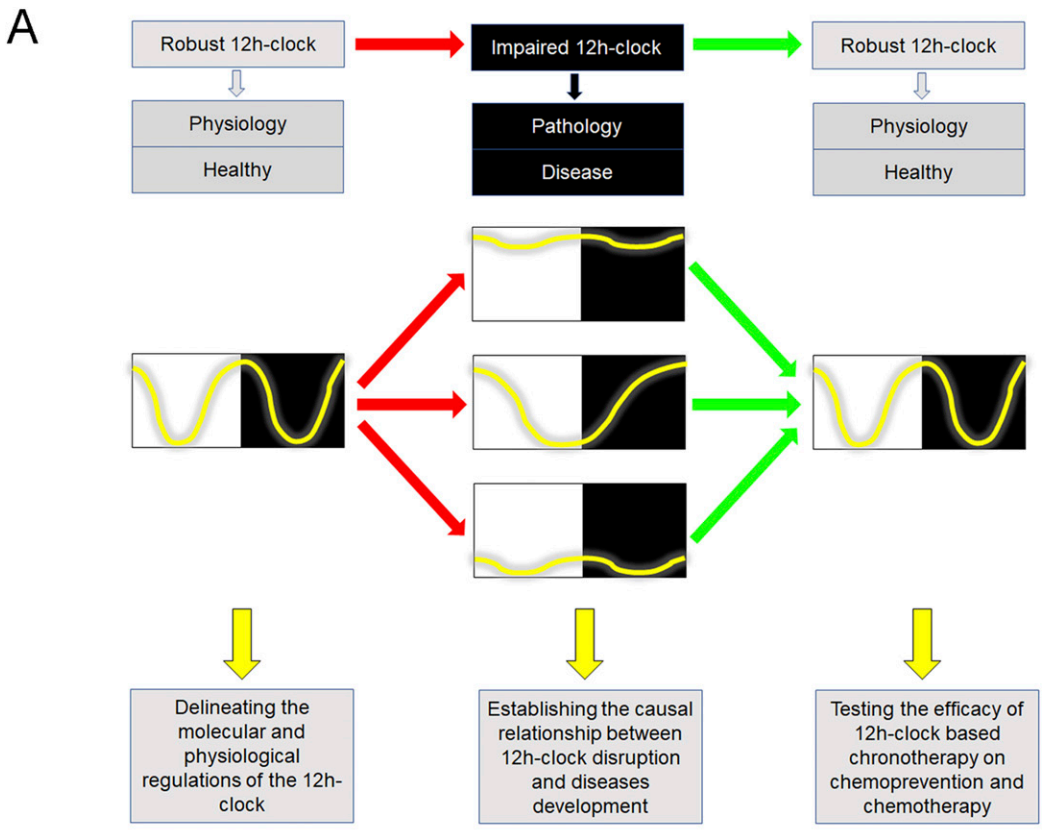
In this section, we present early evidence supporting the potential roles of the 12-hour clock in regulating human diseases and further discuss how we may exploit this knowledge to better implement chronotherapy. Owing to the strong implications that the 12-hour clock is key to the controlling of hepatic metabolism and stress responses, we focus specifically on two conditions that are profoundly influenced by these two pathways: nonalcoholic fatty liver diseases (NAFLD) and aging [125–134].

A. Loss of a Functional 12-Hour Clock Is Strongly Associated With NAFLD Progression

It has been well established that both nutritional challenge and chronic ER/mitochondrial stress are strong contributing factors to the pathogenesis of NAFLD [125, 127, 128, 134–141]. Given the newly discovered housekeeping functions of the 12-hour clock in maintaining cycling metabolic stress response, we propose a new mechanism whereby pathological levels of metabolic stress impair the hepatic 12-hour clock and perturb the metabolic homeostasis that eventually results in NAFLD (Fig. 6A). This hypothesis is reminiscent of the well-established causal relationships among chronic "jet lag stress," and the perturbed circadian rhythm and metabolic syndrome in both animal models and humans [142–145]. At least four pieces of evidence support this hypothesis. First, acute induction of severe ER stress by tunicamycin injection alters the 12-hour rhythmic gene expression in mouse liver [35]. Second, nutritional challenge by high-fat diet severely disrupts the 12-hour oscillation of UPR and metabolic genes, including *Manf*, *Sec23b*, *Hspa5*, *Eif2ak3*, and *Gfpt1* in mouse liver. In contrast, circadian oscillations of all 17 core circadian clock genes, including *Bmal1*, *Per2*, and *Rev-erbα*, were affected either modestly or not at all by the high-fat diet challenge (Fig. 6B) [40]. Thirdly, gene set enrichment analysis revealed that the downregulation of 12-hour gene expressions is associated strongly with progression to hepatic steatosis and nonalcoholic steatohepatitis in humans [3, 146]. Lastly, using both genetic and pharmacological models, it has been convincingly shown that activating ER stress-sensing or ER quality control pathways, including *Xbp1s*, are protective against hepatic steatosis [147–150]. Although this evidence strongly supports an association of 12-hour clock disruption with progression to NAFLD, future studies using both genetic and pharmacological 12-hour clock–deficient animal models are needed to firmly establish the causal roles of the 12-hour clock in disease development (Fig. 6A).

B. Is the 12-Hour Clock in Essence an Antiaging Hormetic Response?

Aging is a complex process characterized by a progressive loss of physiological integrity, leading to impaired function and increased vulnerability and eventually to death. In a


A
Robust 12h-clock
Physiology
Healthy
Impaired 12h-clock
Pathology
Disease
Robust 12h-clock
Physiology
Healthy
Delineating the molecular and physiological regulations of the 12h-clock
Establishing the causal relationship between 12h-clock disruption and diseases development
Testing the efficacy of 12h-clock based chronotherapy on chemoprevention and chemotherapy

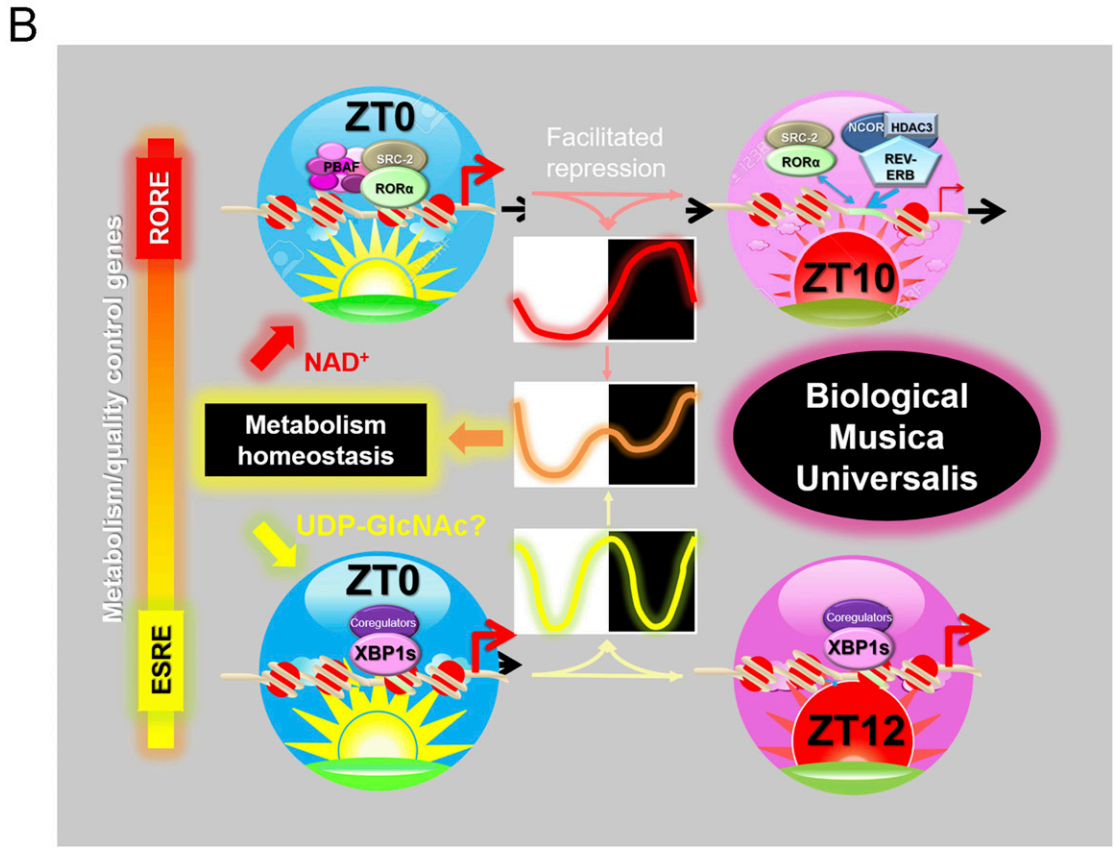

B
Metabolism/quality control genes
RORE
ESRE
ZT0
PBAF
SRC-2
RORα
Facilitated repression
SRC-2
NCOR
HDAC3
RORα
REV-ERB
ZT10
NAD+
Metabolism homeostasis
Biological Musica Universalis
UDP-GlcNAc?
ZT0
Coregulators
XBP1s
Coregulators
XBP1s
ZT12

Figure 6. Framework of 12-hour clock study. (A) Diagram summarizing the potential causal roles of mammalian 12-hour clock in disease development with future directions outlined below. Twelve-hour rhythms can be disrupted either by the dampening of the amplitude or alteration of the period. (B) Diagram summarizing the basis for biological system being part of "musica universalis". ERSE, ER response element; RORE, retinoic acid–related orphan receptor response element.

comprehensive review published in 2013, a total of nine hallmarks representing the common denominator of the aging process in multiple organisms were proposed [133]. Of these hallmarks, loss of proteostasis, deregulated nutrient sensing, and mitochondria dysfunction are biological processes under strong 12-hour clock regulation, suggesting a potential role of the 12-hour clock in mediating aging, especially in the prevention of aging-related metabolic decline [3, 129, 133]. In fact, NAFLD itself is commonly thought to be an aging-related morbidity [134, 151]. Further supporting this hypothesis is the severely disrupted 12-hour rhythm (but not circadian rhythm) of hepatic gene expression in old mice (Fig. 5C) [152], reminiscent of similarly disrupted hepatic 12-hour rhythms in mice fed a high-fat diet (Fig. 5B). Intriguingly, genes known to regulate lifespan in *C. elegans* are highly enriched for 12-hour rhythms of gene expression (Fig. 5D) [75]. These include positive regulators of longevity such as *sir2.1* (homolog of mammalian *Sirt1*) [153–157], *daf-16* (homolog of mammalian *Foxo1*) [158, 159], *aak-2* (homolog of mammalian *Prkaa1/2*, also known as *Ampkα1/2*) [160, 161], and *tcer 1* (homolog of mammalian *Tcerg1*) [162] as well as a large number of negative regulators of *C. elegans* lifespan uncovered from an RNA interference screen [163] (Fig. 5D). More interestingly, *Sirt1*, *Foxo1*, *Prkaa1*, and *Prkaa2* also exhibit 12-hour rhythms in mouse liver (Fig. 5D) [3].

The potential mechanisms of the 12-hour clock in mediating longevity and aging, in our opinion, may lie in the ancient concept of "hormesis," which is the biological embodiment of the idiom "what does not kill you makes you stronger." In scientific terms, hormesis is an adaptive cellular response whereby exposure to low doses of stress (but not a high dose) activates protective mechanisms that render the cell resistant to a subsequent challenge with higher doses of stress [164]. The exact molecular mechanisms underlying hormesis are still unclear but have been previously attributed to the actions of both ER and mitochondria, which are termed ER hormesis and mitohormesis, respectively [124, 165, 166]. In either case, the hormetic response is mediated either by *xbp1*-dependent UPR^{ER} (for ER hormesis) or *atfs-1*/*hsp-60* (*Hspd1* in mammals)–dependent UPR^{mt} (for mitohormesis) [124, 165]. Both ER hormesis and mitohormesis are positively associated with organismal development, stress resistance, and, more importantly, longevity. For example, a study in *C. elegans* showed that expression of XBP1s in neurons was sufficient to increase longevity and induce ER hormetic response in distal, nonneuronal cell types via a cell-nonautonomous mechanism [167, 168]. Furthermore, mitochondrial ribosomal protein knockdown-mediated lifespan extension also requires *hsp60*-dependent UPR^{mt} in both *C. elegans* and mammalian cells [169]. Considering the fact that both ER hormesis and mitohormesis master regulators *xbp-1* and *atfs-1*/*hsp-60* exhibit conserved 12-hour rhythms and the central roles of the 12-hour clock in stress response regulation, we conjecture that the hormetic response may exert its antiaging effects partially through the amplification/boosting of the endogenous 12-hour clock. Indeed, we found that only a low dose, but not a high dose, of tunicamycin (<30 ng/mL) is capable of synchronizing the 12-hour clock in MEFs, consistent with the concept of hormesis (Ref. [3] and unpublished data).

C. Twelve-Hour Clock–Based Chronotherapy

Chronotherapy is the concept that treatment of an illness or disorder should take the body's natural rhythms and cycles into accounts [170]. It has been shown that drugs that target rhythmic, high-amplitude circadian gene products represent a potential pathway for

mechanism-driven chronotherapy [171–175]. Herein we propose that similarly, 12-hour rhythmic transcriptome and proteome can function as a blueprint for future design of 12-hour clock–based chronotherapy. To this end, we probed Food and Drug Administration–approved drugs for potential interactions with high-amplitude 12-hour cycling hepatic transcripts and proteins using the Drug Gene Interaction Database [176, 177] and found a number of hits against both 12-hour cycling hepatic mRNA and/or their protein targets (Fig. 5E and 5F). For example, 6-mercaptopurine is a medication for treating a variety of blood cancer and autoimmune diseases. Among its mechanisms of action is the inhibition of *de novo* purine synthesis by directly inhibiting the rate-limiting enzyme phosphoribosyl pyrophosphate amidotransferase encoded by the *Ppat* gene [178, 179]. Because, as we discussed previously, coordinated 12-hour rhythms of hepatic purine metabolism with RNA transcription is a strong feature of the mammalian 12-hour clock (Fig. 2B and 2C) [3], it may be desirable to administer mercaptopurine (and other drugs targeting *de novo* purine and pyrimidine biosynthesis) at times of nadir purine/pyrimidine metabolism (at 3:00 PM to 4:00 PM) to minimize hepatic toxicity.

Owing to the very early stage of the 12-hour rhythm field (and ultradian rhythms field as a whole), very little is currently known about its definitive functions and implications in human diseases. To a large extent, we can only speculate on their functions and implications based on the limited literature available so far. We hope this review/preview can attract more scientists into this nascent field, because as outlined below, we think that only the tip of the iceberg has been uncovered thus far and that there is so much more to explore.

9. Future Directions

Future work could be directed toward three major aims: (1) delineating the molecular and physiological mechanisms of 12-hour clock regulation, (2) establishing the causal relationship between 12-hour clock disruption and disease progression, and (3) translating the above knowledge into chronotherapy-based medical practice (Fig. 6A). For example, at the molecular and cellular levels, is the 12-hour rhythm temperature-compensated? What is the full spectrum of the 12-hour cycling transcriptome, proteome, and metabolome under 12-hour clock control? What are the negative transcriptional regulators of the mammalian 12-hour clock? What are the coregulators for XBP1s-dependent 12-hour clock transcriptional control? Do some posttranscriptional and posttranslational mechanisms exist? If so, what are they? At the systemic and physiological level, how are the 12-hour rhythms in different tissues and organs coordinated and synchronized at the systemic level? Are there 12-hour cycling hormonal factors coordinating different tissues? Maybe a more tempting question is: does the 12-hour clock exist in the central nervous system and, if so, where? Furthermore, 12-hour clock–deficient animal models are needed in the future to rigorously establish the causal relationship between 12-hour clock dysfunction and multiple disease development. Our ultimate goal should be to successfully translate the newly learned knowledge into chronotherapy-based medicine and disease prevention.

10. Conclusion

Based on the current available literature, we propose a model where combined actions of both the circadian and 12-hour clock are required to ensure cellular homeostasis and promote organism integrity and overall fitness (Fig. 6B). Quite a number of genes in the mammalian genome are under dual circadian and 12-hour clock control, although through distinct molecular mechanisms. The circadian clock is transcriptionally regulated through a revised TTFL model that includes activation phase–facilitated repression mainly via the retinoic acid–related orphan receptor response element (Fig. 6B) [180], while the 12-hour clock is transcriptionally regulated by 12-hour rhythms of XBP1s chromatin recruitment to ER response element (Fig. 6B) [3]. Superimposition of both circadian and 12-hour rhythms of gene expression leads to a temporal gene expression pattern characterized by two nonsymmetrical

peaks within a diurnal cycle (Fig. 6B), which is commonly observed in metabolic genes such as *Fasn* and *Gck* (Fig. 1C) [3]. Furthermore, the circadian clock and the 12-hour clock are synchronized to different environmental cues. The circadian clock is responding to the differential between the two opposite metabolic states, that is, fasting and feeding, whereas the 12-hour clock is entrained by the common denominator associated with both fasting and feeding: the peaking of metabolic stress (Fig. 6B).

In addition to the circadian and 12-hour clocks, it is very likely that additional clock components also exist in mammals, such as an 8-hour clock (Fig. 1B–1D). We reason that having multiple clock components endows organisms with more flexibility and the heightened ability to adapt to different environments, thus markedly increasing survival advantage. In theory, simply adjusting the relative phases and fine-tuning the amplitudes of different clocks can give rise to an infinite number of peak patterns to cope with a variety of daily environmental cycles. The coexistence of multiple harmonic biological rhythms in tune to the natural rhythms is reminiscent of the ancient philosophic concept of "musica universalis," which was first proposed by the Greek philosopher Pythagoras more than 2000 years ago [181]. According to this theory, all natural appearing rhythms are in essence tuned to the rhythmic movements of celestial bodies [181]. Although the circadian rhythm is synchronized to the 24-hour light/dark cycle coinciding with the Earth's rotation, our findings suggest that the 12-hour clock may have evolved from the ancient circatidal clock, which is in turn entrained by the 12-hour tidal cues orchestrated mainly by the moon. Thus, it appears that after 2000 years, we may have finally found evidence for the "biological musica universalis" (Fig. 6B).

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