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## Intracellular and intercellular processes determine robustness of the circadian clock

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### Abstract

Circadian clocks are present in most organisms and provide an adaptive mechanism to coordinate physiology and behavior with predictable changes in the environment. Genetic, biochemical, and cellular experiments have identified more than a dozen component genes and a signal transduction pathway that support cell-autonomous, circadian clock function. One of the hallmarks of biological clocks is their ability to reset to relevant stimuli while ignoring most others. We review recent results showing intracellular and intercellular mechanisms that convey this robust timekeeping to a variety of circadian cell types.

### Introduction

Clocks need to be robust. How useful would a watch be if it was reset by eating lunch or walking to work? Instead, watches are built to withstand and ignore shocks to keep accurate time. However, because no watch keeps perfect time, they need adjusting. The same concepts are true for biological clocks -- they are remarkably robust to perturbation and yet entrain to daily environmental cues such as light and dark. In this review, we explore the underlying basis of the robustness in the mammalian circadian clock to perturbation. Here, robustness is defined as the ability to sustain daily oscillations with an accurate period length day after day. Other studies have highlighted that metrics of robustness depend on how we define circadian performance and at what level (PMIDs: 17158515; 17482552). In oscillatory systems, after perturbation, changes in amplitude, period length, and phase are indicators of robustness, or its inverse, sensitivity. These could relate to the adaptive features of the system to its environment. For instance, jet lag is a byproduct of robust circadian timekeeping, in essence ignoring temporal displacement. Jet lag diminishes, however, due to the system's sensitivity toward light cues; a change in light pattern resets the circadian phase. The ability to maintain robust circadian rhythms in the face of perturbations and uncertainty (e.g. temporal displacement or day length) is a long-recognized and critical property of living systems. Of course, though cellular in nature, physiological disruptions are products of both intercellular and intercellular processes and robustness needs to be considered and measured at multiple levels. In this review, we highlight how this robustness arises from both intracellular and intercellular processes.

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When considering the mechanisms underlying reliable circadian rhythmicity, the plasma membrane naturally divides the processes into intracellular and intercellular. Both processes can differ between cell types. Intracellular processes include the genetic regulatory architecture of the cell. What do we mean by that? We define genetic architecture as the suite of transcriptional and post-transcriptional mechanisms that support normal circadian rhythms. This includes core clock genes such as the E-box activators, *Clock* and *Bmal1*, and their transcriptional repressors, the *Period* and *Cryptochrome* genes. In addition, it includes more recently identified factors such as *Rev-erb-alpha* and *Rora* that function in the clock to regulate levels of *Clock* and *Bmal1*. Intercellular processes include those signaling pathways from one circadian cell which impact oscillations in another. To date, neuropeptides and perhaps gap junctions have been implicated. We will discuss how this architecture changes upon genetic perturbation, and the implications of dynamic network analysis for future study. Finally, we will discuss how recent advances have allowed the exploration of these genetic architectures for the underlying principles that convey robustness.

## Intracellular mechanisms for robustness

### Circadian cells not only respond to environmental change – they anticipate it

The circadian clock pathway is remarkably similar to two other adaptive pathways, the dioxin and hypoxia pathways (reviewed in (1)). While the dioxin response pathway helps mammals to sense and metabolize environmental contaminants, the hypoxia pathway helps mammals respond to low oxygen conditions. Like the dioxin and hypoxia pathways, the circadian clock senses inputs such as light and food. However, in contrast to the dioxin and hypoxia pathways, which are reactive to changes in the environment, the circadian clock anticipates environmental change. All three pathways use bHLH PAS proteins in their signal transduction. As DNA-binding transcription factors, these proteins share remarkably similar response elements, the dioxin response element, a hypoxia response element, and the E-box.

To establish the roles of key players in these pathways, researchers in the circadian, hypoxia, and dioxin signaling fields generated and studied the effect of specific gene knockouts on these pathways. In addition to establishing the roles of these key players in their respective pathways, knockout of many of these genes often resulted in mice with early lethality (reviewed in (1)). In contrast, loss of circadian clock genes (e.g. knockout mice for *Bmal1*, *Clock*, *Npas2*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*) had no effect on survival at birth (2–6). Not only were these mice alive, deficits in circadian regulation of locomotor activity were relatively minor for all but *Bmal1* null mice. How could this be? Circadian clocks are important -- almost all organisms have them -- how could dispensing with key clock components have relatively minor effects on behavior and physiology? Answers to these questions lie in the genetics, biochemistry, and cell biology of the clock, all critical features of its robustness.

### Genetic duplication in the mammalian circadian core loop

The concept of redundancy dominated early thinking on the genetic robustness of the clock. Initial work stemmed from the observation that for every fly circadian clock gene there were typically two or three mammalian paralogs, descendants of a common ancestor of this fly gene (7). Realizing this, several labs created compound homozygous, null mice to test the redundancy hypothesis. First up for these studies were the *Cryptochrome* and *Period* genes. While *Cry1*<sup>-/-</sup> mice had a short period length for locomotor activity in constant darkness, *Cry2*<sup>-/-</sup> mice ran with a long period suggestive that *CRY1* and *CRY2* proteins are not functionally identical (8). However, *Cry1/Cry2* double knockout mice had a much more severe phenotype -- arrhythmicity (8) -- indicating their importance in the clock. The same was true for the *Per* genes. While *Per1*, *Per2*, or *Per3* null mice had minor locomotor

activity phenotypes in constant darkness, *Per1/Per2* double knockout mice were arrhythmic (5, 9). Analysis of *Npas2* and *Clock*, homologs of fly *Clock*, took longer, but the result was the same. Both *Npas2* and *Clock* knockout mice had a mild shortening of locomotor periodicity (3, 10). The compound homozygote *Npas2/Clock* double-knockout mice were again arrhythmic in constant conditions (11). Note: this is condition dependent – Clock mutant mice, which, in contrast to the knockout, retain an altered protein version of Clock, are arrhythmic in constant darkness, but become rhythmic in constant light {PMIDs: 9160755, 9160756, 12465885}. Analysis of *Bmal1/Bmal2* compound null mice was preempted by the observation that *Bmal1* knockout mice were themselves arrhythmic (2). The essentiality of *Bmal1* may in part be explained by its role in regulating its paralog *Bmal2* – when *Bmal1* levels are low, *Bmal2* levels are also low (2, 12). Thus, the clock was by and large tolerant to deletion of its individual components with redundancy as a likely explanation.

### Biochemical mechanism and genetic architecture

But it wasn't that simple. As more biochemical details of the clock were discovered, a surprisingly complex genetic architecture was revealed. To get an oscillation, the clock requires an activator, a repressor, and some mechanism(s) to delay the repressor's action on the activator. Many components of the mammalian clock are transcriptional activators and repressors (Figure 1). Two families of activators, Clock/*Npas2* and *Bmal1/Bmal2*, heterodimerize to bind E-box sequences present in two families of repressors, *Cry1/Cry2* and *Per1/Per2/Per3* (13–14). As message levels of these repressors build up, their proteins are translated and form physical complexes with members of the casein kinase 1 family in the cytoplasm (6, 15–21). After a delay, this repressor complex translocates to the nucleus where it interacts with the activator complex to repress their transcription. This results in lower levels of their own messages. Later in the cycle, these repressor proteins are actively degraded by *Fbx13* and *Btrc* in a proteasome-dependent fashion, thus relieving repression and allowing repressor message levels to accumulate and begin another cycle (22–25). This biochemical mechanism is called the core loop.

But is it one loop or a series of them {PMID: 12620213}? Inherent in this simple mechanism is the capacity for each factor in the clock to regulate the others. For example, *Bmal1*-knockout mice have lower levels of *Per1*, *Per2*, *Cry1*, and *Cry2*, consistent with its role as an activator of these genes (2). Conversely, mice deficient in *Cry1* and *Cry2* have higher levels of *Per1* and *Per2* (8). One consequence of “the core loop” structure, therefore, is the capacity for mutual regulation of gene expression of all components by all components. Although the biochemical mechanism may constitute a loop, the consequence on gene regulatory architecture is a network of E-box loops regulating many core clock genes (Figure 1). In this sense, it may be helpful to think of the core loop as an important motif in the general architecture of the clock network in a cell.

### Evidence for a stabilizing secondary intracellular loop

The circadian clock regulatory structure, however, is composed of more than just the core E-box loop. Schibler and colleagues recognized an ROR element (RORE) in the *Bmal1* promoter (26). This element was identified earlier as a target of two classes of transcription factors, the Rors (activators) and Rev-erbs (repressors). Schibler's group tested a knockout mouse for Rev-erb-alpha and found that, as predicted, *Bmal1* message levels were high (26). In addition, Rev-erb-alpha had an E-box element in its promoter and a high amplitude circadian pattern to its mRNA expression driven by the core loop (27). Later, our group and others showed that *Rora*, an activator of the ROR element, positively activated *Bmal1* expression *in vitro* (28–30). Moreover, *Rora* deficient mice had lower *Bmal1* levels in the suprachiasmatic nucleus (SCN) of the hypothalamus, a population of about 20,000 neurons

critical for circadian rhythms in vertebrates. Mice lacking *Rora* also had short period circadian locomotor activity, a behavior driven by the SCN (28, 30). Because of this mutual regulation, similar to that seen in the core loop, this structure was termed a secondary loop (Figure 1). A similar secondary loop was identified earlier in the fly system, though the fly genes were not related to those found in mammals.

### The role of the secondary loop

The secondary loop reinforces the primary loop by regulating core loop components – this reinforcement confers additional robustness. Mice deficient in *Rev-erb-alpha* were short period length and have a greater dispersion in period length and lower amplitude than wild type littermates (26). A confounding phenotype of the *Rora*<sup>sg/sg</sup> mutation, ataxia, made it impossible to directly link its similar phenotypes to a deficit in circadian clock function (28). Flies or mice deficient in these components have disruptions in gene expression and behavior, but their phenotypes are not as severe as components in the primary loop. Thus, this secondary transcription-translation loop stabilizes, but is not required for, circadian rhythm generation. Moreover, rather than play a direct role in rhythm generation, secondary loop factors seem to act by regulating the expression of components of the core loop.

### Architecture from loops

At nearly the same time, Hiroki Ueda and colleagues began to probe the genetic regulatory architecture of the clock for additional layers of regulation using sequence informatics. They built sophisticated computational models of the E-box, RORE, and D-box (promoter elements bound by PAR bZip factors that were identified as regulators of clock output). In their seminal study, the Ueda group showed that activators and repressors that bind these different elements were mutually ‘wired’ in a web-like genetic architecture (31). The core, E-box loop as well as the ROR and DBP loops were repeated motifs in this genetic architecture (Figure 2). Importantly, in cell-based assays, they went on to show that many of these elements were functional. Thus, while the clock may be composed of core and secondary loops, in action these loops regulate the expression of multiple core clock genes both independently and coordinately. Describing the function of clocks, then, requires an understanding of the higher order genetic architecture in addition to the aforementioned biochemical mechanisms.

### Probing the genetic architecture for characteristics underlying robustness

But how do you study genetic regulatory architecture? Unlike buildings, it’s not easy to see. To get at this issue, our group applied sensitivity analysis and a technique called Gene Dosage Network Analysis (32). In this approach, an individual gene is depleted by RNAi in a dose-dependent fashion generating an “allelic series” of gene dosage. These cells are then put in functional assays to determine the impact of this depletion on clock function. In addition, message levels for other components of the clock system were investigated by RT PCR. If all clock components are truly wired to all clock components, changing any one of them has the capacity to regulate the levels of all others. We did this analysis, and most clock components were found to regulate most others (32) (Figure 2). The finding that several genes are upregulated following knockdown of their paralogs suggests that the clock network utilizes active compensatory mechanisms rather than simple redundancy to confer robustness and maintain function. But there was more. By taking this systematic approach and by collecting experimental data at scale, we were able to investigate this perturbation data for general principles. We found two: paralog compensation and proportional response.

### Paralog compensation: it takes (at least) two, baby

Paralog compensation occurs when one paralog is upregulated in response to deletion of another. This phenomenon has been observed at a genome scale. Pilpel and colleagues analyzed the yeast knockout panel and found that paralogous genes were more likely to respond to a specific knockout than knockouts of unrelated genes (33). It has also been observed in vertebrates (34). In perhaps the most famous case, deletion of the master regulator of myogenesis, *Myod1*, unexpectedly caused little to no phenotype in muscle development (35). Upon investigation, researchers found that its paralog, *Myf-5*, was upregulated in response to deletion of *Myod1* (35). When the double knockout was made, the mice failed to develop normal skeletal muscle and died soon after birth (36). Paralog compensation, rather than simple redundancy, provides a mechanism to ensure gene function upon challenge (i.e. gene loss) and, therefore, conferred robustness.

Our investigation of the circadian system likewise found several examples of paralog compensation, particularly those associated with repressors. For example, depletion of *Per1* by RNAi resulted in up regulation of its paralogs *Per2* and *Per3*. Likewise, depletion of *Cry1* resulted in up regulation of its paralog *Cry2*. Finally, down regulation of *Rev-erb-beta* resulted in up regulation of its paralog *Rev-erb-alpha*. In all three cases, this paralog compensation was unidirectional. In other words, depletion of *Per2* did not produce up regulation of *Per1* and *Per3*, nor did *Per1* and *Per2* respond to depletion of *Per3*. Thus, paralog compensation imparts robustness against some, but not all, perturbations of the genetic architecture. This helps explain how forward genetics has revealed some, but not many, genes in the circadian gene network.

How does directional gene compensation work? It is possible that the paralogs are not wired in exactly the same way; without the right regulatory element, a gene can't respond. Indeed, while *Per1*, *Per2*, and *Per3* have E-box elements in their promoters (what genes don't?), their levels respond differently in *Clock* and *Bmal1* mutant mice (37). Furthermore, they are differentially regulated by light and by the CREB system (38–39). It is also possible that genes are present at vastly different protein concentrations or activity levels. For example, depletion of a less abundant gene may have little or no effect on a more abundant gene, even if it's wired to respond. This may explain tissue-specific robustness. For example, *Npas2* and *Clock* are expressed at similarly low levels in the SCN. Loss of one or the other yields a modest circadian phenotype, perhaps due to paralog compensation, whereas loss of both abolishes circadian behavior (11). In contrast, *Npas2* is expressed at high levels in the neocortex, where it has been speculated to play a more important role in circadian timing than *Clock* (40). In liver, *Npas2* levels are elevated in response to *Clock* knockout; however, the liver clock is arrhythmic (3, 51). Interestingly, there is no evidence for cell-autonomous circadian timing in these brain areas (41), suggesting that cell-specific changes in the relative abundance of clock genes may not always support circadian clock function. This needs further examination, especially given the recent report that *Per1* and *Per2* are not always found in the same cells, at least in brain (42). Thus, tissue specific expression and other perturbations change the genetic architecture of the clock and consequently its function.

### The principle of proportionality

Perturbation of the clock by Gene Dosage Network Analysis showed that the oscillator can produce three types of proportional responses: linear, inverse, and nonlinear. For example, depletion of *Bmal1* resulted in strictly linear transcriptional responses of *Rev-erb-alpha* and *Rev-erb-beta* levels: deplete *Bmal1* levels by 50%, *Rev-erb-alpha* levels drop by 50%. These responses can also be sub-linear, but proportional: deplete *Clock* levels by 50%, *Rev-erb-alpha* levels drop 25%. Responses can be disproportionate: deplete *Cry1* and *Cry2* levels by



50%, and *Per2* and *Rorc* levels increase by 11 times and seven times respectively. This is by no means a clock-specific principle. Similar proportionality has been observed in both yeast and higher eukaryotic systems (34). Thus, this network sensitivity analysis uncovered how simple principles of paralog compensation and proportionality were built into the circadian oscillator.

## Intercellular mechanisms for robustness

### Hiding weaknesses in a network

One of the most remarkable recent discoveries is that cell-cell interactions can make circadian oscillations more robust against a wide variety of perturbations including genetic mutations. The success of genetics in circadian biology depended on easily measured changes in the stable, repeatable daily rhythms of locomotor activity. In mammals, forward genetics revealed a role for the *Clock* gene in the determination of circadian periodicity of locomotor activity in mice (43). The discovery of *Clock* came quickly (the first *Clock* heterozygote was mouse #25 in the mutagenesis screen for altered circadian locomotor patterns). So it's surprising that the subsequent 15 years of mutagenesis screens yielded only one additional central regulator of circadian periodicity, the *Fbxl3* gene (44–46). Forward genetics in flies was more productive and offered candidates that, when knocked out in mice, revealed roles for genes including *Per1*, *Per2*, *Cry1*, and *Cry2*. The regulatory architecture in mice appeared to protect against loss of key clock components, where flies, which lack clock gene paralogs, had more severe phenotypes.

More recently, studies of the cellular basis for the behavioral deficits in these mice revealed that the coupled SCN network is more resistant to genetic perturbations than single SCN neurons. For example, the *Clock* mutation produces arrhythmicity in the firing patterns of dispersed SCN neurons, but a long period in SCN slices and behavior (47). Similarly, dispersed SCN neurons from *Cry1*<sup>-/-</sup> or *Per1*<sup>-/-</sup> mice exhibit weak circadian rhythms in PER2::LUC activity but high amplitude rhythms in SCN slices (48). These results are consistent with analyses of chimeric, *Clock*-mutant mice which showed that lengthening of circadian period and loss of amplitude correlated with a decreasing proportion of wild-type to *Clock/Clock* cells. Strikingly, the circadian pacemaker mechanism does not suddenly break down with the introduction of some *Clock*-mutant cells (49). Thus, cellular network interactions can protect against some genetic deficits. This provides a warning to geneticists who give up when they see no behavioral phenotype in their knockout mouse; cellular communication could mask the loss of function that may be apparent in single cells.

The same idea holds for peripheral clocks. Imaging analysis of liver, lung, cornea, or fibroblasts from *Cry1*<sup>-/-</sup> mice showed an arrhythmic phenotype, while behavioral rhythms or those from SCN slices were short period length (48). Lung and fibroblasts from *Per1* knockout mice are arrhythmic, while behavioral analysis of the same mice show short period length (48). While *Rora*<sup>sg/sg</sup> mice are short period length in locomotor behavior, fibroblasts from these mice are arrhythmic (50). In contrast to *Clock*<sup>Δ19/Δ19</sup> mice, which have a mutation that causes exon skipping and loss of 51 amino acids in the Clock protein and are long period for locomotor activity, *Clock*<sup>-/-</sup> mice, with no expressed exons, have short period length behavioral rhythms. Lung and liver samples from these knockout mice are arrhythmic (51). Thus, behavioral rhythms and intact SCN slices from circadian clock gene knockout mice are protected against the more severe phenotypes seen in cell autonomous preparations from their periphery.

### Sloppy clocks made precise

The idea that single cells are unstable circadian clocks made robust by network interactions also holds for wild-type SCN neurons. We recently found that fully isolated SCN neurons

can express circadian rhythms in gene expression or firing rate, but their rhythms are low amplitude and their periods are unreliable (52). Dispersed SCN neurons show about 10 times greater cycle-to-cycle period variability than those in the connected SCN explant (53). In SCN slices, blocking action potentials (and presumably intercellular communication) with tetrodotoxin (TTX) also results in sloppy circadian rhythms of gene expression in many neurons (54). Furthermore, individual cells in SCN slices that were arrhythmic in a primary TTX treatment can remain arrhythmic or regain rhythmicity in a subsequent TTX treatment (52). This ability to generate and lose rhythmicity appears to be common across multiple classes of cells in the SCN. This is reminiscent of how neurons that fire in rhythmic bursts can transition to tonic firing patterns with small changes in their various conductances depending on a combination of intrinsic and network properties (55). In circadian cells, perhaps small changes in the molecular clock (e.g. degradation rates of PER2) result in cells which fall off their limit cycle and become unstable.

Remarkably, Rat-1 fibroblasts are not unstable, but instead appear to oscillate with a relatively stable, cell-autonomous period for at least 15 days (56). This is similar to the precise circadian timing of individual cyanobacteria, which do not appear to share circadian timing information with each other (57). Among mammalian cells, SCN cells appear to be unique in their ability to synchronize to each other.

### **Intercellular signals that improve robustness**

One of the signals responsible for the synchrony and precision among SCN cells has been identified -- vasoactive intestinal polypeptide (VIP). VIP is secreted in a circadian pattern from a subset of approximately 10% of SCN neurons (58). Loss of VIP or its receptor results in mice that have trouble entraining to a poorly lit day, one hour of light at dawn and another at dusk (59). In constant darkness, the locomotor activity of most of these mice is arrhythmic or comprised of multiple circadian periods (59–61). SCN from these mice match their behavioral phenotypes with no rhythm at the population level, although a subset of individual cells express desynchronized, unstable circadian rhythms in firing rate or gene expression. A minority of mice deficient in VIP signaling can express a shortened free-running period in behavior and in the SCN (59–61), suggesting additional signaling can coordinate the ensemble. These other coupling signals have not been identified, but candidates include other neuropeptides (e.g. gastrin releasing peptide or vasopressin) and communication through gap junctions. Even signals such as glucocorticoids or body temperature, acting indirectly or directly on SCN rhythmicity, could play a role in this robustness against the loss of the primary intercellular coupling factor, VIP (62–64).

### **How do intercellular signals stabilize intracellular circadian signals?**

Ultimately, they need to act on the molecular oscillator. Although little is known about the specific transduction pathways, we and others have hypothesized that cell-cell signaling increases the amplitude of the circadian oscillator, reducing its sensitivity to perturbations (65). For example, robustness could result from amplification of transcription of critical clock genes (Fig. 3). In this respect, VIP increases cAMP (66–67), requires protein kinase A (PKA) to phase advance firing rate rhythms in the SCN (68), and can induce transcription of *Per1* and *Per2* in a CREB-dependent manner (69). Other transmitters, hormones, gap junctions, or even temperature changes in the body, could similarly entrain and augment *Per* gene transcription in local or global populations of circadian cells (70).

### **Relevance of robust rhythmicity to reality**

To be useful a circadian clock must keep accurate time so that it can control processes in anticipation of daily changes in physiology and the environment. It must also be adjustable

to local time and, thus, seek a balance between robustness and adaptability. Therefore, there may be conditions when circadian oscillators are naturally less robust.

### Neonatal SCN may be less robust

SCN from newborn rodents express circadian rhythms in gene expression, metabolism, and firing rate, but do not control daily rhythms in behavior for several more days to weeks depending on the model and behavior. Recently, Nishide et al. showed that SCN from postnatal day 6 mice phase shift more than 8 hours to stimuli that have no effect on adult SCN (71). It may be that developmental changes in molecular oscillator components or intercellular connectivity regulate the responsiveness of the circadian clock to perturbations. This would suggest that, for whatever reasons, neonatal rhythms are particularly susceptible to environmental disruptions.

### Times to throw out a clock

Arctic reindeer and voles may turn off circadian oscillations as an adaptive advantage in their unusual lighting environments. Recently, Loudon and colleagues showed that fibroblasts from reindeer either have extremely low amplitude rhythms or have lost autonomous clock function altogether (72). It remains to be seen how changes in intracellular state, SCN connectivity, or their combined effects mediate some of the developmental or seasonal adaptations in mammalian circadian rhythms (73).

### Future directions

Recent work has raised the possibility that inputs outside the core clock can influence or even substitute for its function. For example, cell metabolism (i.e. redox state) can be under circadian clock control and also regulate transcription of key clock genes (74–75). Similarly, cAMP levels oscillate with time of day and can shift circadian gene expression (76). In SCN context, intercellular signals likely modulate ATP and cAMP levels, a path from network effects to single-cell circadian oscillations. On a systems level, body temperature, under the control of the SCN, can influence circadian timing in peripheral tissues and in the SCN (62–63). How and whether these intracellular and intercellular signals protect the clock against environmental perturbations remains to be determined.

Traditional, ground-up modeling will also play an important role. Much of our recent progress has benefited from using cell autonomous models of the clock. These models are convenient in that they allow for high throughput analysis of clock gene perturbations, and indeed, even whole genome screening for clock modifiers. They are also more amenable to biochemical approaches than the SCN. Indeed, much of the biochemistry that fuels ground-up models of the clock comes from either the cell autonomous models or easily studied peripheral tissues such as the liver. As the field moves further, understanding the tissue specific biochemical parameters of the clock will become important. There isn't one clock; there shouldn't be one clock model.

Finally, the nascent field of network biology is just beginning for the clock. In that regard, the transcriptional translational feedback loop may have gotten us here, but its utility is waning in explaining the complexities of clock biology. As future studies uncover more about the clock network architecture, we may consider these biochemical loops as motifs in larger structures that explain the complexity of clock biology. While the circadian gene network has begun to give up its principles, the features that make it so robust will be the hardest nuts to crack



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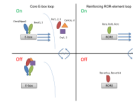
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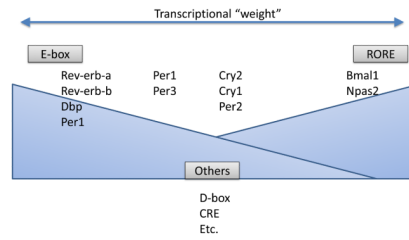


**Research highlights**

- Circadian systems oscillate in a noisy environment and entrain to daily timing cues.
- Their robustness against environmental and genetic perturbations depends on their biochemical and genetic architecture.
- Paralog compensation, transcriptional response programs and balanced biochemical feedback loops help sustain daily cycling.
- Robustness also arises from intercellular (e.g. neurotransmission) and systems-level signals (e.g. body temperature).

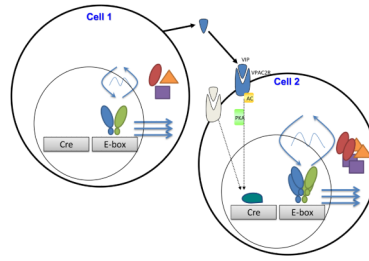
**Figure 1.**

Biochemical engines and regulatory architectures. The two primary biochemical engines of the clock are the E-box and RORE regulators. (Top) A simplified diagram of these engines. In the upper panels, a theoretical “100% on” state is depicted. Clock and Bmal1 bind to E-box elements recruiting the basal PolII machinery and activating transcription. Rora, Rorb, and Rorc likewise bind to their response elements, the RORE, to activate transcription from structural genes containing these elements. (Bottom) In the lower panels, a theoretical “100% off” state is depicted. In the case of the E-box, the repressor complex (Cryptochrome, Period, and casein kinase 1) has translocated to the nucleus, bound to the Clock/Bmal1 complex, and repressed the complex possibly by disrupting their DNA-binding potential. In the case of the RORE, Rev-erb-alpha and Rev-erb-beta displace the Ror factors to actively repress transcription by recruiting histone deacetylases.



**Figure 2.**

The contribution of response elements to clock gene expression is depicted. Certain genes are dominated by E-box input, e.g. Rev-erb-alpha or DBP. Other genes are dominated by RORE input, e.g. Bmal1. Many genes integrate input from both elements, e.g. Cry1 or Per2. Other elements also contribute – the CRE and D-box elements are notable examples. In this model, like a synaptic weight, all genes have the potential to get input from all transcriptional complexes, but do so to a greater or lesser extent depending on the complex.



**Fig. 3.**

Schematic of putative network pathways to amplify the intracellular circadian oscillator. Because the loss of VIP or its receptor leads to fewer high amplitude circadian cells, we illustrate how intercellular VIP may increase the amplitude of intracellular gene expression rhythms. Cell 1 expresses the E-box based molecular oscillator shown in Fig. 1. Cell 2 expresses the same molecular oscillator and receives a signal (VIP) which acts through its receptor (VPAC2R) to activate second messengers (e.g. adenylyltransferase and protein kinase A) to ultimately enhance clock gene expression (e.g. phosphorylated CREB binding to the CRE promoter element on the clock gene or another interacting gene). The increased transcription results in more clock gene products at the time of their peak production and a higher amplitude oscillation. The same perturbation on these two cells would be predicted to have a larger effect on the oscillations of Cell 1. This highlights that intercellular signaling both increases robustness and provides points of sensitivity in the architecture underlying reliable circadian cycling. Future studies will consider how these pathways may change with age, may adjust to the seasons, or may be impacted by modern, non-circadian lifestyles.