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Circadian Oscillators: Around the Transcription-Translation Feedback Loop and on to Output

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Abstract

From cyanobacteria to mammals, organisms have evolved timing mechanisms to adapt to environmental changes in order to optimize survival and improve fitness. To anticipate these regular daily cycles, many organisms manifest near 24-h cell-autonomous oscillations that are sustained by transcription–translation-based or post-transcriptional negative feedback loops that control a wide range of biological processes. With an eye to identifying emerging common themes among cyanobacterial, fungal and animal clocks, some major recent developments in the understanding of the mechanisms that regulate these oscillators and their output are discussed. These include roles for antisense transcription, intrinsically disordered proteins, codon bias in clock genes, and a more focused discussion of post-transcriptional and translational regulation as a part of both the oscillator and output.

Keywords

Clock; TTFL; PTO; Intrinsically Disordered Protein; Codon Bias; Antisense

Highly conserved circadian systems

Most organisms are subject to the dramatic day-to-night oscillations of the environment on Earth. Species have evolved circadian rhythms to optimize their cellular metabolism to be in tune with this regular change. Circadian rhythms in every organism are cell autonomous, appear to have arisen only a few times in evolution, and can be driven by one of a few lineage-specific but otherwise highly conserved central oscillators. While oscillators driving bacterial and plant clocks are distinct from each other and from other known clocks, fungal and animal cells share circadian oscillators of conserved regulatory architecture:

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transcription-translation feedback loops (TTFLs) comprised of two parts. Specifically, 1) a positive arm with a heterodimeric complex at its core that behaves as the activator of the system, promoting the transcription of 2) one or more components of the negative arm, which when translated inhibit the activity of the positive arm [1, 2].

The circadian field has exploded in recent years with newly discovered clock-regulated organisms and clock functions. However, basic clock mechanisms have been consistently studied in just a few species; it is from these model systems that most discoveries elucidating basic circadian paradigms derive. Cyanobacteria, which utilize both a TTFL and a Post-Translational Oscillator (PTO), provide an interesting “outgroup” for comparison with the comparable TTFL systems used by animals and fungi, seeing in this way how evolution has solved similar problems of circadian timing and output in very distinct groups of organisms. (See Box 1, Box 2, and Figure 1 for a basic overview of circadian clocks in model organisms). Illumination of the circadian clock mechanism is far from stationary, and this review identifies and embraces some of the emerging controversies regarding how the clock operates and how new roles for clock proteins affect both the models of how the clock keeps time and also how the circadian system benefits its host. There have been many recent circadian advances, indeed more than can be summarized here, and so we attempt to find common ground among systems at the expense of discussing organism-specific advances. In doing so, we highlight two overarching areas characteristic of circadian systems in general: 1) the negative arm and its regulation of the core clock; 2) the control of output by the positive arm and its environment.

New developments in the Negative Arm Function

In this section, we focus on the negative arm, examining 1) insights into possibly conserved roles for antisense transcription; 2) the increased awareness of the role of structure for negative arm proteins; 3) the interaction between codon bias and protein expression in negative arm regulation; and 4) the emerging idea that turnover may not be essential for setting period length or closing the feedback loop.

Antisense regulation of negative arm expression

Antisense RNA is emerging as an important factor influencing the core operation of the negative arm of the clock. Expression of *Neurospora qrf*, the long non-coding antisense RNA of *frq*, oscillates in antiphase to *frq* mRNA [3]; *qrf* contributes to both phase and resetting of the clock. A mammalian *Per2* antisense transcript also oscillates antiphasic to *Per2* [4, 5]. *qrf* expression is light-induced through the action of the White Collar Complex (WCC, Box 2) and is also constitutively expressed via an unknown light-independent mechanism [6]. The function of *qrf*, the prototypic circadian antisense molecule, is currently an area of much interest and discussion. A recent report by Xue et al. observed that inhibiting the expression of *qrf* amplifies the expression of *frq* mRNA, causing the organism to become arrhythmic [6]. Their model postulates that H3K36me3 chromatin modification, as well as premature transcription termination resulting from the stalling/collision of the RNA polymerases transcribing *frq* and *qrf* [7], lowers the troughs of expression of *frq*. This ensures that *frq* and FRQ are only expressed in robust synchronous waves and creates a

double negative feedback loop within the core of the *Neurospora* circadian feedback loop. Loss of convergent transcription allows leaky FRQ expression at trough times and results in loss of synchronicity and rhythm damping [6].

Focusing on a completely different premise, that *frq* and *qrf* RNAs could form sense-antisense hybrids and lead to formation of facultative heterochromatin, Li et. al. explored the effects of regulated *qrf* expression [5]. Under this model, *qrf* expression would act initially to activate, creating a transcriptionally permissive state at the *frq* locus, and later to repress by promoting formation of heterochromatin. This is consistent with the observation that *qrf* enhances m⁵C methylation (an important epigenetic gene silencing mechanism) at *frq* [8].

Both models encompass the idea that antisense expression silences sense expression. They also allow that chromatin modifications can occur while polymerase collision affects sense RNA levels (Figure 2A), although the facultative heterochromatin model is vague as to how dicer-independent siRNA (disiRNA)-mediated recruitment of heterochromatin enzymes occurs. With both *Neurospora* and mammalian cells containing potentially regulatory antisense transcripts, it is likely that the fine-tuning of negative arm proteins by an antisense RNA is a conserved mechanism of regulation. These reports set the stage for what is likely to be a longer experimental thread with broad relevance to understanding eukaryotic circadian oscillators.

Negative arm structure dictates function

Although there are clear evolutionary links among the positive arm Per/Arnt/Sim (PAS)-domain-mediated heterodimeric complexes (Box 2), links that extend past the divergence of fungi and animals [9, 10], one of the surprising and disconcerting bits of information about the molecular clockworks has been the relatively poor conservation among the negative arm proteins. Whereas the positive arm components in *Neurospora*, WC-1 and WC-2, are seen throughout the fungi, FRQ appears more sporadically outside of the ascomycetes [11–14]. Among animals, TIM is essential for the *Drosophila* clock but has no apparent role whatsoever in mammals while CRY, the essential negative arm repressor of the mammalian clock, is a photoreceptor in *Drosophila* with no apparent role in the oscillator but can be a repressor in other insects [15]. What are the common features that link negative arm proteins together?

One such feature, oddly, is the PAS fold (Box 2) [16, 17]. Although now better known structurally as the prominent feature of the positive arm heterodimer partners, for some time these PAS domains were the only structurally well-defined regions of the negative arm clock components. Circadian-associated PAS domains have been crystalized in some clock-model organisms and are known to play a role in the interaction between the clock proteins [16–22]. However, most negative arm proteins (e.g. FRQ, CRY, FRH) have no PAS domain; even when the PAS domain is present (as in PER) it accounts for only a small fraction (<20%) of the total length. The remaining sequences encode a mix of alpha helices, beta sheets, coiled-coil domains and, interestingly and in nearly all cases, poorly ordered regions and stretches of low sequence complexity that often contain abundant sites for posttranslational modifications.

Despite the lack of structure, these regions do not lack importance, and a recent thread in the circadian literature has described roles for these disordered regions. For example, the core negative arm proteins in the *Neurospora* clock, FRQ and FRH, contain unstructured regions [23–27]. The unstructured regions of FRQ are so large that the protein is considered an Intrinsically Disordered Protein, or IDP [25, 26]. As an IDP, FRQ is at risk for degradation by default [28]. To avoid this fate FRQ interacts with its stabilizing binding partner FRH [26, 29]; without this interaction with FRH, the half-life of FRQ is cut by two thirds and clock function is ablated [26, 29] (Figure 2B). Of note, the interacting regions of both FRH and FRQ are predicted to be highly unstructured which may help with their interactions [26].

Discernment of this FRQ-stabilizing role for FRH in the clock was entirely unexpected given that FRH is a highly ordered protein [26], and is an ATP-dependent RNA helicase with an entirely distinct and essential role in the cell [30]. As this theme of dual non-overlapping roles for FRH was emerging, contrasting work based in *in-vitro* studies asserted that FRH played a more active role in the clock, via its ATPase activity, by stimulating a structural change in FRQ [31]. However subsequent analyses have shown that this ATPase-dependent structural change seems to be specific to the *in vitro* yeast system used for the experiments and does not occur in *Neurospora* (personal communication from M. Brunner). This is consistent with published findings that the amino acids within FRH that are required for ATPase and helicase enzymatic function are not required for circadian function [26]. More definitively, the complete structural determination of FRH identifies notable structural differences between FRH and its yeast ortholog MTR4p that signal its unique circadian function [27]. This paradigm wherein FRH plays an essential non-enzymatic but structural role in the negative element complex has potentially broad impact because the PERs in mouse and human cells are also predicted to be highly unstructured [26]. Interestingly, in cyanobacteria, the N-terminal ATPase domain of KaiC, which is structurally homologous to FRH, is needed to allow refolding of KaiB to permit assembly of KaiB-KaiC complexes [32, 33]. Therefore, one could expect that negative arm proteins in higher eukaryotes, like FRQ, will have stable binding partner(s) that confer on them both structure and stability.

Codon Bias is essential to proper negative arm structure

Given that inherent structure is unimportant for the proper functioning of FRQ, it is surprising that the structure and stability of FRQ is highly sensitive to the codon usage of the gene [34]. FRQ displays a strong bias for low usage codons that is conserved and also appears essential for proper functioning of the clock; FRQ is so sensitive to codon bias that optimizing the codon usage in just the N-terminal end of FRQ disrupts clock function. The interpretation is that by increasing the speed of translation elongation, FRQ may not properly fold, thus it cannot enter into essential interactions with other proteins and is unable to perform the functions necessary to complete a circadian cycle [35]. In support of this, when the codon-optimized FRQ is expressed at low temperatures (and therefore translated at a lower rate) its resistance to freeze/thaw cycles and proteases is similar to that of wild type FRQ [34]. However, the few domains of FRQ predicted to be highly structured have both high usage codons and low disorder and are less sensitive to what codons are used, and thus appear not to require slow translation to fold [36]. An alternative explanation to the theory that negative arm proteins need slow translation to fold is that too rapid translation of FRQ

may interfere with the binding of its stabilizing partners, which include FRH and CK1 [34], thus leading to rapid degradation-by-default of FRQ and loss of clock function [26].

In contrast to arrhythmicity resulting from codon optimization of FRQ, codon optimization of the cyanobacterial clock gene, *kaiC*, affects neither period nor phase [37]. Unlike FRQ, KaiC is predicted to be highly structured and has only slight codon bias. In fact, when the codon usage in the *kaiBC* operon of *S. elongatus* was optimized to mimic that of highly expressed genes resulting in increased Kai protein expression, rhythms were enhanced at cooler temperatures. Surprisingly however, the improvement in the strength of the rhythms was accompanied by a very apparent loss of fitness compared to strains bearing normal (non-optimized) *kaiC* genes. When normal and optimized strains were grown at cooler temperatures under entraining Light:Dark cycles, the cooler temperatures induced a long period in the optimized strain and, as circadian entrainment theory predicts, entrainment of a long clock to a 24 hr day leads to a rhythm with a significantly delayed phase compared to the normal strain [38]. The result is that clock-controlled metabolic processes no longer happen at the correct time of day. This is maladaptive, and thus, the “optimized strains” with the strongest rhythms did not grow as well as WT. This codon bias-mediated suppression of rhythms under certain conditions (“circadian conditionality”) shows that at times a robustly running clock may actually be a disadvantage to an organism.

Negative arm protein turnover isn't essential for closing the circadian feedback loop

While studies of factors affecting the translation of negative arm proteins have been salient in the literature, perceptions of the role of degradation of the negative arm proteins have also been changing. Past research had suggested that in fungal, insect and mammalian clocks, being degraded was the final clock-relevant action of the negative arm proteins. This was believed to set the period of the clock [24, 39–44], hence the current model in which the positive arm drives expression of negative elements, which repress the activity of their activators until they are turned over, thus releasing the positive elements to begin a new cycle. Indeed, there is a long-standing and excellent correlation between negative arm protein half-life and period length seen in both *Neurospora* and *Drosophila* [39, 45].

This theory is also consistent with a variety of data from strains bearing mutations in the proteins that target other proteins for degradation, as well as inhibitor studies. For instance, in *Neurospora* strains in which the F-box protein FWD-1 (which mediates proteasomal degradation of FRQ) is deleted, FRQ turnover is impaired and overt conidial rhythms are ablated [46]. In animals, mutation of orthologs of FWD-1 stabilizes PER proteins and lengthens period, as do mutations in proteins that target CRY for turnover [44, 47–50]. Recently however, and unexpectedly, by tracking the molecular cycles in the clock using a luciferase reporter fused to FRQ protein, molecular circadian rhythms of FRQ[LUC] activity were seen in strains lacking FWD-1 and in which bulk FRQ levels (as shown by He and Liu [46]) remained nonrhythmic and elevated [51]. Similar results were seen in strains where the COP9 signalosome (which also plays a role in the degradation of FRQ [52]) was deleted [51]. These results demonstrate that the degradation of FRQ, a canonical negative arm element, is not essential for maintenance of a functional clock. This evidence eliminates degradation as a determinant for setting day length and leaves FRQ phosphorylation as the

main determinant. The model emerging from Larrondo et al. [51] envisions two kinds of clock protein phosphorylation, one specific to determining period (presumably by influencing protein structure, perhaps nuclear localization, and interactions within the oscillator) and the other leading specifically to degradation [23, 24, 51]. If the two kinds of phosphorylation proceed in parallel, period length and stability will be correlated as has been seen; only by compromising protein turnover can the impacts of the two kinds of phosphorylation be made distinct (Figure 2D). In mammalian clocks, changes in stability of mouse CRY1 elicited by mutating the degradation machinery, or even by altering phosphosites within CRY1, do not correlate well with changes in period length [48, 53]. In addition, period length changes due to mutations that affect the stability of PER and CRY are simply additive and do not reflect the epistatic relationships predicted by the existing model [54]. In total, these data suggest that the model of Larrondo et al. may apply to animal clocks as well. Thus, clock protein half-life no longer predicts circadian period length, and instead the emerging data promise a new synthesis for explaining the long time-constant of the clock.

Negative arm repression of transcriptional activity

New data show that the mammalian PER proteins act as scaffolds to recruit a large cohort of effector proteins with varying functions. Many of the proteins that interact with PER are either general transcriptional suppressors with very little specificity [55–58] or are RNA binding proteins such as helicases [55–58], some of which are orthologs of clock-affecting proteins in *Neurospora* [59]. To bring about a repressive chromatin environment, the PER complex actively recruits enzymes driving epigenetic modifications, including histone deacetylases [55] and methylases [56]. Interestingly, this is facilitated by CLOCK/BMAL1 itself which, when bound to and activating the *PER* genes, promotes monoubiquitination of histone H2b thereby covalently marking the chromatin for later recruitment of the repressive PER complex [60].

While much attention has been focused on factors promoting de novo transcription initiation (discussed later), the majority of transcripts in cells arise from transcription reinitiation within already active chromatin. Another aspect of repression is that the PER complex preferentially accumulates near the termination sites of *Pers* and *Crys* where it sequesters proteins needed for reinitiation [57]. A very nice codicil to these studies reveals how some of these interactors, members of the NuRD complex, play a role in the specificity of transcriptional suppression in mammals. This is achieved through splitting the NuRD complex components between the PER and the CLOCK-BMAL1 complexes [61]. In order for repression to occur by histone deacetylation, each half of the NuRD complex must come into contact through the interaction of the PER and CLOCK-BMAL1 complexes (Figure 2C). Future work is likely to identify additional factors and show in greater mechanistic depth how they and the existing players achieve repression.

The Positive Arm and Clock Output Regulation

In this section, we examine the regulation of circadian output by the positive arm, focusing on 1) the role of the positive arm as a Pioneer transcription factor to change heterochromatin

in order to allow secondary transcription factors (TFs) to regulate gene expression; 2) posttranscriptional regulation of clock output and; 3) new evidence of the responsiveness of the clock to the environment.

The positive arm incorporates a Pioneer transcription factor

The long standing model of the role of positive arm proteins in the clock is that they activate the expression of both the negative arm components as well as the expression of *ccgs*, genes that are directly or indirectly regulated by the positive arm but that do not play a role in the core clock complex [1]. However, there is an inconsistency in this model in *Neurospora* and in mammals, as the heterodimeric activator (i.e., CLOCK:BMAL1 or WC-1:WC-2) has a peak in activity at only a single phase of the cycle yet *ccgs* peak throughout the circadian day. Thus, there have to be other elements involved in the phasing of Output [62–65]. A similar phasing issue arises in the cyanobacterial system (Box 2) but surprisingly, the disconnect between a single heterodimeric activator peak and the multitude of *ccgs* peaks is not observed in *Drosophila*, where binding of the core transcription factor aligns closely with the peak of transcription of circadianly expressed genes [66].

This paradox in fungi and mammals was resolved by the demonstration that the heterodimeric circadian activators promote the rhythmic removal of nucleosomes from DNA [67, 68]. In this way, the circadian activators act as “Pioneers” [69], opening up virgin chromatin territory for the subsequent binding of other TFs. Rhythmic chromatin opening at sites bound by CLOCK:BMAL1 or WC-1:WC-2 may facilitate the rhythmic binding of other TFs, and the activity of these secondary TFs contributes to the temporally heterogeneous transcriptional output of the positive arm target genes [62, 64, 67] (Figure 3A). Elements responsible for rhythmic epigenetic modifications and chromatin remodeling include CSW-1, CHD-1, and CATP in *Neurospora* [8, 70, 71]. Most recently, work in *Neurospora* has demonstrated that the WCC calls down the SWI/SNF complex to remodel chromatin at the “clock box” within the *frq* promoter, thereby facilitating the DNA bending needed to effect circadian activation from this distal region while not affecting *frq* light regulation [68]. These data challenge the idea that positive arm protein binding alone sets the phase of, and activates, core clock gene transcription. It is clear that other TFs and/or mechanisms collaborate with positive arm binding and are critical to determine the phase of clock-controlled gene transcription. The identification of these additional factors and description of the regulatory hierarchy in which they operate is a significant thread in ongoing work in eukaryotes, following the nice elucidation of output pathways governing circadian gene expression in cyanobacteria ([72]; see Box 1).

Post-transcriptional regulation is extensive in circadian output

In the simplest scenario, transcriptional activation of *ccgs* could lead directly to rhythms in processed transcripts yielding slightly phase-delayed rhythms in clock-controlled transcripts. Instead, however, there is often a great deal of post-transcriptional regulation of circadian output. Data from *Neurospora*, flies and mouse liver all demonstrate poor correlation between the activation of a promoter and the amount of the corresponding transcript for genes that are rhythmic at the steady state level [4, 63, 64, 73] (Figure 3B). In mammalian cells, comparison of RNA-seq data with data either from large scale Nascent-seq [63] or

transcriptional profiling [4] identified many genes that are rhythmic at the transcriptional level but not at the steady state level and vice versa [4, 63]. In flies, similar discrepancies were noted and a link between post-transcriptional regulation and the amplitude of rhythms was also demonstrated [73, 74].

In the case of fungal clocks, this topic is still under debate. When promoter activation was tracked using fusions between potentially rhythmic gene promoters and luciferase, a significant discordance was discovered between genes that are rhythmic at the mRNA level and those that are rhythmically activated. This was even the case when gene-specific 3' untranslated regions (3'-UTRs) were included with the reporters in an attempt to mimic more accurately all regulation [64]. A later study compared transcriptional profiling (via RNA-seq) with RNA polymerase II occupancy (via chromatin immunoprecipitation-sequencing, ChIP-seq) to study rhythmically transcribed genes [65]. Although samples were taken only over the first day in darkness, the first 8 hours of which are still heavily influenced by the prior LD cycle, these data showed greater synchrony between transcript levels and transcription but still revealed that about a third of genes did not show rhythmic abundance in sync with expression. Sancar et al. [65] suggested that some of this discordance could be due to the stability of the transcripts, or simply to difficulties in detecting poorly expressed mRNAs. However, given that such a large percentage of *ccgs* described both by Hurley et al. [64] and by Sancar et al. [65] show discordance between levels of activation and expression, it is unlikely that rhythmic transcriptional regulation alone is sufficient to completely describe how the core clock can control circadian output in fungal clocks as well as in higher eukaryotes.

One novel and potentially important aspect of circadian post-transcriptional regulation is RNA transmethylation, which affects translational regulation [75] and has been suggested to control every step of the central dogma of biology [76]. When transmethylation is inhibited biochemically, or through specific knockdown of the methyltransferase *Mettl3*, the length of the circadian period increases in the liver. There are many predicted sites of methylation on clock gene transcripts, any of which could be responsible for the long period phenotype in transmethylation-repressed cells [75].

The circadian clock is responsive to the environment

While historically most circadian research has focused on the core mechanism, circadian control of transcription is the principle means through which the clock exerts control over an organism and is therefore a key piece to understanding the functional relevance of the clock. Depending on the organism, anywhere from 5% to nearly 100% of the genome is under the control of the circadian clock, making understanding what the clock is regulating an integral part to understanding the advantage of maintaining a biochemical circadian oscillator [64, 77, 78]. A major aspect of clock control in *Neurospora* is metabolic output and recent work has identified the major metabolic KEGG and FunCat pathways that are enriched in genes under circadian control [64, 65]. Gene products involved in catabolism, energy production and precursor assembly are in the dawn peak of circadian transcription while the dusk expression peak is enriched in more downstream pathways like macromolecular biosynthesis, ostensibly to concentrate more efficiently processes within the most beneficial

phase of the day [64, 65]. Not surprisingly, cellular metabolic regulation imparted by the clock is also seen in cyanobacteria (discussed later) and in mammals [79].

Interestingly, and reflecting the adaptive significance of the clock, circadian output is flexible in response to an organism's metabolic environment. This was revealed by Hurley et al [64] who compared a subset of circadianly regulated genes from *Neurospora* grown on two different nutrient sources; significant differential expression was noted, with specific subsets of genes being rhythmically activated in one media or the other but not both. As the clock was running normally in both cases, this indicates that the environment, in this case the metabolic environment, is able either to feed back through or to integrate into an as yet unknown mechanism to regulate the spectrum of *crgs* without affecting the core clock function [64].

In cyanobacteria, global output is transmitted to the master controller for Output, RpaA, in two ways, via SasA (which phosphorylates and activates RpaA) and via CikA (which dephosphorylates and inactivates RpaA). The histidine protein kinase SasA interacts with KaiC and autophosphorylates, then transfers the phosphoryl group to RpaA. In opposition to this, the phosphatase activity of CikA is activated when CikA binds to KaiB, opposing the action of SasA [32, 80]. Phospho-RpaA binds to 110 genome locations, activating circadian dusk-specific gene expression. This RpaA regulon includes sigma factors and other secondary effectors, some effecting changes in DNA topology, that collectively transform the fixed dusk-peak/dawn-trough RpaA activity cycle into a continuum in which expression of genes can peak at any time throughout the circadian day [72]. RpaB, which is light-dark regulated, may also contribute to phasing. Absent a clock, the default state for Output is a high level of phospho-RpaA so, perhaps counterintuitively, the principle Output of the clock is dawn-inhibition of RpaA. This reduces catabolic processes such as the pentose-phosphate cycle and frees up carbon for other biosynthetic activities [72, 81], leading to anabolic processes peaking near dawn [81]. Thus, like *Neurospora*, cyanobacteria use their clock to partition metabolism as a function of time but do so in the opposite phase from *Neurospora* where daytime metabolic potential favors catabolism, energy production and precursor assembly whereas night favors macromolecular biosynthesis and growth (discussed earlier). This difference in phasing makes sense for a photosynthetic lifestyle in which the critical anabolic factors, ATP and reductant, are driven by light.

Concluding remarks

Recent years have seen a surge in the molecular analyses of both the output and core mechanisms of circadian clocks. Yet, as often happens, with greater comprehension come new questions and many of these key questions are evident from this recent work. For example, Larrondo et al. [51] have shown that in the *Neurospora* system, the clock runs normally even when negative arm protein half-life is severely affected. Although there are data consistent with this conclusion in animal clocks, this hypothesis has yet to be rigorously tested. In addition, it is unclear if the clock-specific phosphorylations of the negative arm proteins are the sole determinant of period or if there are other elements that play a role in setting the period of the clock. Along the same vein, the lack of structure demonstrated in the *Neurospora* clock protein FRQ appears, at least at the sequence level, to be conserved in

higher eukaryotes and this raises the question of why circadian negative arm proteins are so often largely unstructured. A better understanding of negative arm component protein structure may provide insight into the method of communication between the positive arm and the negative arm and the actual mechanism of repression of positive arm function.

Outside of the core mechanism, mechanistic details of the benefits of clock regulation are coming into focus. Compensation is an emerging theme; that is, how can changes in the ambient environment affect clock output while not affecting the core mechanism? Analysis of circadian output is becoming more prominent. Circadian control of transcription has been considered the principal means of output in all systems, so elucidation of the hierarchical networks governing this will inform both biology and human health [82]. Circadian control beyond the transcriptome is advancing due to improvement in techniques [83]; ribosome profiling is revealing new aspects of post-transcriptional regulation of output. How output changes and how outputs such as metabolism can feed back to influence the core oscillator is set to be a compelling mystery for the clocks field in the coming years.

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Box 1: A Primer on the Prokaryotic Circadian System

The cyanobacterium *Synechococcus elongatus* is the model bacterial clock. The central Post-Translational Oscillator (PTO) is composed of only three proteins: KaiA, KaiB, and KaiC, [84]. KaiC is both an autokinase and autophosphatase that can be phosphorylated at two positions [85]. The phosphorylation state of KaiC determines the phase of the clock in the organism [86]. KaiA promotes KaiC autokinase activity while KaiB negates KaiA's action and promotes KaiC dephosphorylation [87]. Thus the rate of ATP hydrolysis, which is extremely slow compared to typical ATPases, is an important determinant of clock period. Interconnected with the PTO is a TTFL (Transcription Translation Feedback Loop) oscillator based on daily clock-controlled expression of the *KaiBC* operon. Most studies agree that the PTO (now reconstituted in another cell type [88]) is the necessary-and-sufficient core oscillator. However, even absent the KaiABC-based PTO, the TTFL is capable of driving temperature-compensated rhythmic gene expression (albeit with a long 48 hr period length) of a substantial portion of the genome [89], suggesting that while the PTO is required for robustness and proper periodicity it may cooperate with the TTFL for output. Two recent analyses have tied the slow rate of ATP hydrolysis in the PTO to rates of structural changes in the Kai proteins, via a slow peptide isomerization in KaiC [90] and a slow and rare shift between folded states of KaiB that affects its ability both to capture KaiA (and thereby promote KaiC dephosphorylation) and to bind to and activate CikA, thereby influencing output [32].

Recent work in cyanobacteria has clarified how Input to this circadian system works. Unlike fungal and animal clocks where light is perceived by dedicated photoreceptors, light resetting in cyanobacteria works exclusively via photosynthesis and metabolism [91]. Prior work showed that light-driven changes in energy charge or oxidized quinones are sufficient to reset the clock [92, 93] but did not eliminate metabolism-independent Input pathways. Pattanayak et al. [91] demonstrated that when obligate photoautotrophic strains were engineered to grow in the dark on glucose, they were rhythmic and blind to light exposure, suggesting that all clock resetting in cyanobacteria works through light-driven metabolism. In the cyanobacterial clock, Output feeds back to Input when the TTFL drives circadian expression of the Kai genes and RpaA, thus supplying non-phosphorylated KaiC in a cyclic manner on a daily basis [94] [95]. In addition, daily synthesis of quinones and starch may reinforce the core oscillator [81, 93].

Box 2: A Primer on Fungal and Animal Circadian Model Systems

Although totally unlike clocks in cyanobacteria or higher plants, fungi and animals use clocks that display a degree of similarity that is perhaps remarkable given the broad evolutionary distance encompassing these organisms; then again, perhaps this is not so surprising as animals and fungi share a common ancestor more recently than either does with any other kingdoms. The core oscillator in fungi and animals is a Transcriptional-Translational Negative Feedback Loop (TTFL) in which delays between the appearance of clock proteins (via translation) and their subsequent action in effecting negative feedback constitutes most of the long 24 hour time constant [1, 41]. This TTFL is often supported by coupled feedback loops that lend robustness as well as providing Output. Any delayed action negative feedback loop, the circadian oscillator included, must have an activating positive arm and a repressing negative arm. In fungi and animals the positive arm consists of two proteins that interact (via PAS domains) to form a DNA-binding heterodimeric transcriptional activator that drives expression of the negative arm proteins. PAS domains, named after Per, Arnt, and Sim, are short ~50 amino acid domains that are associated with signaling proteins and often bind a ligand [96]. In *Neurospora*, the positive arm proteins White Collar-1 and White Collar-2 form the heterodimeric White Collar Complex (WCC); in *Drosophila* the heterodimer includes CYCLE (CYC) and dCLOCK (dCLK), and in mammals BMAL1 and CLOCK. In *Neurospora* the WCC promotes expression of the negative arm protein Frequency (FRQ) which interacts with FRQ-Interacting RNA Helicase (FRH) and Casein Kinase 1 (CK1) to form the repressive complex that interacts with WCC to depress its activity [97, 98]. In *Drosophila* CYC-dCLK drive expression of the negative arm proteins PERIOD and TIMELESS which complex with DBT, a *Drosophila* CK1, to form the repressive complex [99], and in mammals the heterodimeric BMAL1-CLOCK complex positively regulates expression of negative arm component genes, the *Periods* and *Cryptochromes* (encoding PER1, PER2, PER3, CRY1 and CRY2), that combine with CK1 and several other proteins to make the repressive complex that depresses BMAL1-CLOCK activity [100].

Light Input to fungal and animal TTFLs begins with dedicated photoreceptors that elicit signaling that acts to induce (in fungi and mammals) or reduce (insects) the amounts of negative arm proteins [1, 41]. In broad outline, Output occurs when the Positive Arm heterodimer binds to DNA and activates expression of genes whose products do not impact the TTFL.

Outstanding Questions Box

1. What is the nature of the transcriptional network controlling circadian output in eukaryotes, and what are the links influencing the conditionality of circadian output in different environments?
2. Antisense transcripts are seen for several genes encoding proteins central to negative arm functions. What role(s) do antisense transcripts play, and are these role(s) conserved?
3. Both phosphorylation and protein-protein interactions are cited as means through which the positive arm represses the negative arm. What is the relative contribution of negative arm protein-mediated phosphorylation of positive arm proteins compared to protein interactions? How universal are the existing mechanisms describing how the negative arm effects repression on the positive arm?
4. How important are post-transcriptional regulations in the overall circadian regulation of the proteome?
5. Is there a universal common mechanism for circadian temperature compensation among eukaryotes, and if so how does it work?

Trends Box

1. An apparent lack of structure is a common feature of some of the negative arm proteins in fungi and animals, and may be essential to their proper function in the circadian clock.
2. The mechanism of circadian period determination does not lie in the half-life of the negative arm proteins, leaving the potential for alternative methods, such as post-transcriptional regulation, to fill that role.
3. Transcriptional regulation of clock-controlled genes *ccgs* by the heterodimer in the positive arm, once believed to be the absolute driver of changes in expression of *ccgs* and of output, appears to be just the first step in the pathway of circadian control over cellular output.

Figure 1.

Minimal schematic representations of circadian model systems. A. Cyanobacteria. The Post Translational Oscillator (PTO) composed of KaiA, B, and C is seen at the top and output from it, which drives the Transcription Translation Feedback Loop (TTFL) as well as clock-controlled genes (*ccgs*), is at the bottom. Briefly, the core PTO oscillation derives from the autokinase-autophosphatase activity of KaiC. Kai A promotes the autokinase, and Kai B promotes the autophosphatase activity and traps KaiA into inactive complexes, crucial for sustained oscillations, before they are both lost from the KaiC complex. Output from the PTO proceeds through the kinase SasA and (via KaiB) through the phosphatase CikA; among the loci controlled is the *KaiBC* operon, thereby constituting a TTFL that supplies KaiB and KaiC. See text and Box 1 for details. B. Fungal and Animal Clocks. TTFLs comprising heterodimeric activators (blue ovals) and common parts of negative elements complexes (red ovals, deliberately simplified) are the core circadian oscillators. Their generalized actions within the clock and in driving output are shown below: Positive Elements (PEs) drive expression of the gene(s) encoding the Negative Elements (NEs). After translation these NE proteins are slowly post-translationally modified, eventually reaching a state where they become effective in forming a repressive complex that depresses the expression of the own gene(s). Although additional post-translational modification of the NEs leads to their degradation this does not contribute to the timing mechanism. The daily cycle of repression and de-repression of the PEs creates a means for output from the clock when the PEs drive expression of *clock-controlled genes*. Stars represent phosphorylations; the pink oval represents inactivated/degrading complex. See text and Box 2 for details.

Figure 2.

Recent advancements in elucidating negative arm function. A. Negative arm noncoding antisense RNA creates a double negative feedback loop via either ribosome collision or creating a transcriptionally permissive state. B. Stability of an Intrinsically Disordered Protein (IDP) in the negative arm (red oval) is enhanced by its partner protein (grey moon), stabilizing the IDP until it is able to impart its function in the clock. C. Negative arm repression is conferred, in part, by negative arm proteins acting as a scaffold to bring into contact transcriptional repressors (green elements), whose components may be split between the positive arm and negative arm. D. Degradation of the negative arm proteins is not essential to release the clock from a repressive state. Likely, clock-specific phosphorylation regulates negative arm activity and degradation-specific phosphorylation regulates degradation of the negative arm half-life. Blue ovals represent the positive arm complexes, red ovals represent the negative arm complexes, stars represent phosphorylations; the pink oval represents inactivated/degrading complex, green shapes represent transcriptional repressors, orange ovals represent ribosomes, grey moons represent Nanny proteins.

Figure 3.

Current models of Positive Arm operation. A. The heterodimeric transcription factor complex of the Positive Arm in fungi and mammals has been shown to act as a Pioneer transcription factor (TF), in some cases allowing secondary TFs to set the phase of *ccgs*. B. Post transcriptional mechanisms regulate the output of the clock, making it possible for genes that are rhythmically activated to be arrhythmic at the steady state mRNA level, as

well as genes that are not rhythmically activated to be rhythmic at the mRNA steady state level.

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