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The circadian system as an organizer of metabolism

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Abstract

The regulation of metabolism by circadian systems is believed to be a key reason for the extensive representation of circadian rhythms within the tree of life. Despite this, surprisingly little work has focused on the link between metabolism and the clock in *Neurospora*, a key model system in circadian research. The analysis that has been performed has focused on the unidirectional control from the clock to metabolism and largely ignored the feedback from metabolism on the clock. Recent efforts to understand these links have broken new ground, revealing bidirectional control from the clock to metabolism and vice-versa, showing just how strongly interconnected these two cellular systems can be in fungi. This review describes both well understood and emerging links between the clock and metabolic output of fungi as well as the role that metabolism plays in influencing the rhythm set by the clock.

Keywords

Circadian; Metabolism; *Neurospora*; Clock-Controlled Genes; Output

Circadian clocks

Circadian rhythms are biological cycles with a period of a single day (circa [around] diem [dies or day]) that persist in the absence of time cues but are still able to be reset by them. A central oscillatory mechanism controls both the length of the circadian day as well as the regulation that is imparted by the clock. This regulation, or output, adjusts innumerable behaviors affecting everything from sporulation in *Neurospora* to sleep in humans. Clocks in fungi and animals have an oscillator comprised of two parts: 1) a positive arm, typically a heterodimeric complex that acts as the activator of the cycle, promoting transcription of the second component; 2) the negative arm, which when translated is able to inhibit the activity of the positive arm (reviewed in (Dunlap, 1999)).

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Circadian clocks are a phenomenon conserved from cyanobacteria to humans (Bell-Pedersen et al., 2005); in rhythmic environments, organisms having clocks with period lengths close to those in the environment outcompete arrhythmic strains, demonstrating the advantage these clocks convey to the organisms that maintain them (Dodd et al., 2005; Ouyang et al., 1998; Woelfle et al., 2004). Many of the advantages that are associated with the clock are conveyed through the clock's link to metabolism, which allows the organism to optimize its daily output to better anticipate circadian environmental changes. Core components of the mammalian circadian clock are directly involved in the promotion of genes involved in metabolism (reviewed in (Bass, 2012; Eckel-Mahan and Sassone-Corsi, 2013; Sancar and Brunner, 2014)). In addition, interconnected molecular feedback loops involving both the clock and metabolism have been demonstrated in many higher eukaryotes and the mis-synchronization of these cycles can lead to effects on almost all organismal systems (reviewed in (Bass, 2012)).

Circadian Clocks in Fungi

Though the circadian clock is present in organisms from cyanobacteria to humans, the fungal clock, particularly that of *Neurospora crassa*, has been an important model for how circadian rhythms are maintained. A filamentous fungus, *Neurospora* is originally best known for the one gene, one enzyme hypothesis (Beadle and Tatum, 1941). With the manifold similarities between the *Neurospora* and animal circadian systems, a fully sequenced and well annotated genome (Galagan et al., 2003), facile recombineering with 98% efficiency, functional genomics, a genome scale metabolic model, a high throughput knock out project (Colot et al., 2006; Dreyfuss et al., 2013; Dunlap et al., 2007), and the development of a CRISPR system (Matsu-ura et al., 2015), *Neurospora* has become one of the most tractable model organisms for the study of chronobiology at the level of the cell.

The positive arm of the clock in *Neurospora* is comprised of a heterodimeric transcription factor complex, the White Collar Complex (WCC), made up of White Collar-1 (WC-1) and White Collar-2 (WC-2) (Figure 1). WC-1 and WC-2 interact via PAS domains and drive the expression of a salient protein in the negative arm of the clock, Frequency (FRQ) (reviewed in (Fuller et al., 2014; Hurley et al., 2015)). FRQ binds to its partner protein Frequency Interacting RNA Helicase (FRH) immediately upon translation and this interaction stabilizes FRQ, as FRQ is an Inherently Disordered Protein (IDP) and is intrinsically unstable absent a partner (Cheng et al., 2005; Guo et al., 2009; Hurley et al., 2013; Querfurth et al., 2011; Shi et al., 2010; Zhou et al., 2013). The FRQ/FRH complex (FFC) combines in a stable manner with CK1 and interacts with the WCC to suppress the production of *frq* mRNA (reviewed in (Hurley et al., 2015)). FRQ has no known enzymatic function but is believed to act as a protein scaffold, bringing the components of the oscillator together, consistent with the finding that FRQ is an IDP.

Within the fungal lineage, in addition to the well-demonstrated core clock in *Neurospora*, many of the core clock proteins are conserved in other species (Dunlap and Loros, 2006; Fuller et al., 2014; Salichos and Rokas, 2010). Components sufficient to assemble complete circadian feedback loops are seen universally in the Sordariacea and beyond, suggesting that many plant and animal pathogens have a functional clock. So it is not surprising that the

only other molecularly characterized fungal clock has been reported in *Botrytis cinerea*, including both the positive arm utilizing the WCC as well as a negative arm involving FRQ (Canessa et al., 2013; Hevia et al., 2015). Interestingly this clock plays a role in the virulence of the organism (Canessa et al., 2013; Hevia et al., 2015).

The positive elements of the clock (WCC) and other clock components (FRH, kinases and ubiquitinating enzymes) have been found in a wide array of fungi, including Zygomycetes, Basidiomycetes, and Ascomycetes, but the clock-exclusive protein FREQUENCY (FRQ) is less conserved. Until recently, FRQ had only been seen in Sordariomycetes, Leotiomycetes, and Dothideomycetes (Salichos and Rokas, 2010). However, new data suggests that FRQ is more conserved than originally believed as an orthologous FRQ has been identified in *P. confluens*, the last common ancestor of filamentous ascomycetes (Traeger and Nowrousian, 2015). In Saccharomycetes both the WCC and FRQ were lost in what appears to be a genome size reduction and these yeasts have never been demonstrated to possess circadian rhythms (Dunlap and Loros, 2006; Salichos and Rokas, 2010).

Outside of the demonstration of a molecular clock, rhythms have been reported in other fungi, including conidiospore formation in the Zygomycete *Pilobolus* (Bruce et al., 1960), as well as growth and developmental rhythms in a variety of Ascomycetes including *Aspergillus* spp. (Dunlap and Loros, 2006; Greene et al., 2003). More recently, bioluminescence rhythms have been demonstrated in the basidiomycete *N. gardneri* in which the bioluminescence helps the organism to draw in insects, which aids in the circulation of spores (Oliveira et al., 2015). Given that rhythms have been reported in *Aspergillus*, which has no FRQ, it may be that rhythms in these organisms have a different negative arm protein but still use the same positive arm complex, the WCC (Dunlap and Loros, 2006; Greene et al., 2003).

Circadian Output

The benefit of conserving a functional molecular clock is theorized to be that it gives the cell information on the time of day and allows the cell to better regulate its output in tune with its environment. This time of day response is achieved through the primary output of the clock, consisting of a subset of rhythmically expressed genes termed the clock-controlled genes, or *ccgs*. A great deal of effort has been concentrated in attempting to identify these *ccgs* and the role they play in regulating cellular function. The initial screens for clock-regulated genes were carried out with subtractive hybridization (Loros et al., 1989) and identified just two *ccgs*. One was a fungal hydrophobin and the other a glucose repressible gene, which in addition to being clock regulated was also regulated by light, and oxygen tension. Larger scale and more sensitive differential screens identified further *ccgs* (Bell-Pedersen et al., 1996) but it was not until EST and microarray technology was developed that *ccgs* in the clock could be tracked at global levels (Correa et al., 2003; Dong et al., 2008; Nowrousian et al., 2003; Zhu et al., 2001), identifying hundreds of potential *ccgs* of which many were linked to metabolic output. However, recent improvements in technology have allowed for *ccgs* to be more thoroughly and sensitively tracked using RNA-seq, demonstrating that as much as 40% of the *Neurospora* genome could be regulated by the circadian clock (Hurley et al., 2014; Sancar et al., 2015).

As is seen in higher eukaryotic systems (Koike et al., 2012; Menet et al., 2012), there is a great deal of posttranscriptional regulation on the *Neurospora* clock (Hurley et al., 2014). Promoter activation does not directly correlate with mRNA steady state levels and this downstream regulation has a potentially large effect on the circadian system, as only about 25% of investigated genes were rhythmic at both the expression as well as the mRNA steady state level (Hurley et al., 2014). There are many likely avenues that could account for these differences, including some key methods of posttranscriptional regulation that have been demonstrated in higher eukaryotes (reviewed in (Brunner and Schafmeier, 2006; Lim and Allada, 2013)). In the *Neurospora* clock, posttranscriptional regulation has been shown in a few cases, including the regulation of the transcript of *frq* by the FFC and exosome as well as the expression of an antisense to *frq*, *qrf* (Guo et al., 2009; Li et al., 2015; Xue et al., 2014).

The Circadian Clock in *Neurospora* controls metabolic output

While previous work has hinted that the link between the circadian clock and metabolism might be significant, high throughput RNA-seq analysis shows the extent to which this regulation occurs may be far greater than originally believed (Figure 2). When the genes that are regulated by the clock at the level of mRNA are interrogated by gene ontological (GO term) analysis, genes that fall in metabolic pathways are highly enriched. In fact, among the GO categories appreciably enriched in genes regulated by the circadian clock, nothing is more significantly enriched than those pathways involved in metabolism (Hurley et al., 2014). Examining the data further, most major categories of cellular metabolism display some rhythmic control as well as the majority of ontological subcategories.

These RNA-seq data sets also point to a phasing of metabolic output that is regulated by the clock. Metabolism genes are highly enriched in the late circadian evening to the early circadian morning but are not enriched in the circadian afternoon (Hurley et al., 2014). Genes up-regulated in the circadian afternoon and evening are less involved in metabolic output but are enriched in pathways like protein synthesis. It appears that the distinct dusk and dawn phasing of the clock organizes the molecular output of the cell so that catabolic reactions occur in the morning while anabolic reactions occur at dusk (Dong et al., 2008; Hurley et al., 2014; Sancar et al., 2015). For example, genes involved in ribosome biogenesis and translation peak in the circadian dusk to early evening while genes involved in downstream functions involving proteins, i.e. protein folding, targeting, and modification, peak in the early circadian morning. This temporal separation of physiology is most likely synchronized in order to confer even further efficient regulation of available resources as well as to take advantage of or protect from environmental conditions.

Metabolic feedback on the circadian clock

Based on the RNA-seq data, many of the genes controlled by the clock play a role in metabolic output. Perhaps more interestingly, some of these genes actually function to feed metabolic information back into the clock. One of these, and perhaps the best studied, is Conidial Separation Protein-1 (CSP-1). *csp-1* is a *cgc* directly targeted by the WCC (Lambreghts et al., 2009; Smith et al., 2010). The closest homologues to CSP-1 are the yeast

transcriptional repressors NRG1 and NRG2, which are regulated by glucose (Berkey et al., 2004). CSP-1 is also a Zn-finger transcription factor that can act as a circadian repressor (Sancar et al., 2011). Genes regulated by CSP-1 are repressed in the circadian morning and activated in the circadian evening, suggesting that CSP-1 plays a role in properly timing the expression of circadian output genes (Sancar et al., 2015; Sancar et al., 2011).

In addition to its role in regulating the output of the clock, CSP-1 helps the clock respond to the metabolic environment of the organism. Clock periods in a *csp-1* strain decrease with increasing amounts of carbon sources (glucose, sucrose or fructose) while periods in WT strains are unaffected by changing glucose levels (Sancar et al., 2012). This observation demonstrates that CSP-1 plays a role in the compensation of the clock against metabolic conditions. Further analysis shows that CSP-1 can regulate the mRNA levels of the WCC; when glucose levels are high, CSP-1 represses the expression of *wc-1* mRNA while transcription of *wc-1* is de-repressed when glucose levels are low. This compensates for the fact that overall rates of translation of WC-1 are high when glucose is high, buffering the clock against increasing translation rates in high glucose. CSP-1 achieves this because it is itself regulated by glucose levels, with CSP-1 increasing in amount under high glucose conditions. These higher levels increase repression at the *wc-1* promoter, and thus buffer the clock against high levels of glucose in the environment, compensating for the increased levels of metabolites.

A connection between metabolism and circadian output has been demonstrated outside of CSP-1 regulation. Lipid metabolism, which is also regulated by CSP-1 (Sancar et al., 2011) and is enriched in clock output according to GO analysis (Hurley et al., 2014), can feed back on the clock to affect period length at the level of the core oscillator. For instance, high levels of sn-1,2-diacylglycerol (DAG) are suggested to feed back on the clock to lengthen periods (Ramsdale and Lakin-Thomas, 2000). In addition, the double mutant of the *lag-1* and *ras-1* genes shows defects in cell wall lipid rafts (Case et al., 2014). Both *lag-1* and *ras-1* have been shown to regulate the life span in *Neurospora* as well as in humans (Case et al., 2014; Jazwinski et al., 2010). A combination of the *lag-1* and *ras-1* mutations are reported to inhibit conidiation rhythms in *Neurospora* and lengthened FRQ period to 41 hours (Case et al., 2014). Finally, a strain bearing the *prd-1* mutation has been shown to be defective in clock compensation against glucose (Starkey et al., 2014).

Clock output adjusts to metabolic conditions

There are several well-studied mechanisms of input into the circadian clock. The response to light, for which *Neurospora* is also a model organism, is perhaps the best studied and the most understood of the clock inputs (reviewed in (Fuller et al., 2014)). In addition, temperature has been shown to reset the phase of the clock as well as effect the period (reviewed in (Hurley et al., 2015)). The third known input into the clock, metabolism, has long been studied from the perspective of the clocks regulation of metabolic output and the buffering of the clock against changes in the metabolic environment. However, a major development in understanding the link between metabolism and the circadian clock is that while the clock is buffered at its core against the metabolic environment, the clock still recognizes environmental changes and finetunes which genes are activated at the level of

output to adjust to these differences. This was demonstrated by tracking the output of the clock in two different growth media, one with high levels of glucose and arginine and the other with low levels of glucose and arginine as well as the addition of Quinic Acid (Hurley et al., 2014). It was noted that while all genes were expressed in both assay conditions, for some of the genes, rhythms were only detected in one of the two media conditions (Hurley et al., 2014). 50% of the genes were rhythmic in both media and when subjected to GO analysis; these genes were enriched in core cellular functions including cell cycle, cell transport, and protein fate. However, genes that were rhythmic in only one of the two media were enriched specifically in the metabolic GO categories (Hurley et al., 2014).

These data suggest two possibilities; 1) the rhythmicity of genes involved in core cellular functions is more stringently controlled, ensuring little to no change in circadian regulation due to changing environment; or 2) there are multiple loops feeding back on the clock and only the subsets that were enriched in metabolic GO categories are responsive to the metabolic feed back. Genes from other GO categories would be responsive to the feed back from other loops specific for distinct conditions. Other influences, such as suppression of expression as well as posttranscriptional modifications are unlikely as they were either controlled for or assayed directly and eliminated during the experiment. While the answer to this riddle is unclear and most likely is a combination of the two possibilities mentioned above, what is undoubtedly demonstrated is that the clock is not just a static master controller that with each cycle expresses blanket metabolic genes in anticipation of any circumstance, but is actually a flexible link between the environment and the transcriptome. Circadian regulation must be plastic in its control of the gene expression hierarchy to best suit the needs of the organism in its current environment.

Conclusion

Clock control has always been thought to be an extensive process involving a large amount of the genome. However, the recent evidence linking the clock to metabolic output demonstrates just how important and broad this link really is. As an organism of potential importance for biomass conversion, understanding the role that the clock can play in *Neurospora* is essential. On the flip side, understanding how the metabolic environment feeds back on clock output can teach us a great deal about how the clock effects adjustments to an organism's environment. These interlocking regulatory loops (circadian and metabolic) provide an excellent model for understanding how systems within a cell interact in a way to benefit the organism.

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Figure 1.

The *Neurospora* molecular circadian clock. In the night, aging and highly phosphorylated FRQ releases the WCC from its inhibited state and FRQ is subsequently degraded in a process whose kinetics do not affect timekeeping. Post-translational modifications on WCC are reversed and WCC induces expression of *frq* mRNA, leading to high levels of FRQ translation shortly after dawn. Nascent FRQ binds to FRH as well as forming homodimers and stably interacts with CK1. Several kinases, chief among them being CK-1, phosphorylate FRQ, influencing its structure and interactions. The FRQ/FRH/CK1 complex inhibits WCC activity probably by promoting the phosphorylation of the WCC. Loss of WCC activity leads to a decrease in FRQ synthesis. Meanwhile old FRQ is increasingly phosphorylated, leading to its decreased affinity with and final dissociation from WCC; this allows reactivation of the WCC starting the cycle anew. Ubiquitination of old hyper-phosphorylated FRQ, facilitated by FWD-1, directs FRQ degradation.

Figure 2.

Physiological control of development by the circadian clock. The core clock mechanism, (represented by the blue loop), drives the activity of the WCC. WCC activated genes (represented in red, with a few examples named) drive the development of the organism by activating a network of transcription factors (TFs). In turn, metabolic conditions feed back on to the output of the clock via largely unknown mechanisms (green lines). The core clock is buffered against the effects of changing metabolic conditions through the action of CSP-1 (shown in orange) and other factors yet to be described. However, both light (yellow, sensed by WC-1) and temperature (purple) can affect the clock directly.