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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 vise-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.

1. 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)).

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) and 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)).

2. 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) (Fig. 1). WC-1 and WC-2 interact via PAS domains and

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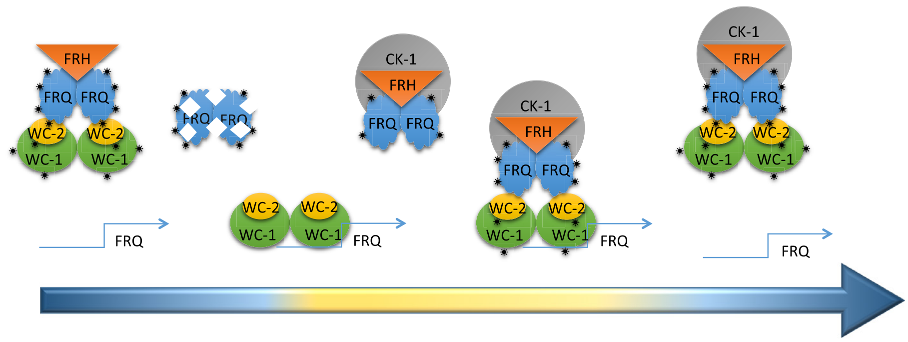

Fig. 1. The Neurospora molecular circadian clock. In the night, aging and highly phosphorylated FRQ releases the WCC from its inhibited state and FRQ is subsequently degraded in a process whose kinetics do not affect timekeeping. Post-translational modifications on the WCC are reversed and the WCC induces 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 the WCC; this allows reactivation of the WCC, starting the cycle anew. Ubiquitination of old hyper-phosphorylated FRQ, facilitated by FWD-1, directs FRQ degradation.

drive the expression of a salient protein in the negative arm of the clock, Frequency (FRQ) (reviewed in Fuller et al. (2014) and 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)), in total creating the archetypal transcription-translation circadian negative feedback loop. 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 (the 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).

3. 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