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Principles of the animal molecular clock learned from Neurospora

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Abstract

Study of Neurospora, a model system evolutionarily related to animals and sharing a circadian system having nearly identical regulatory architecture to that of animals, has advanced our understanding of all circadian rhythms. Work on the molecular bases of the Oscillator began in Neurospora before any clock genes were cloned and provided the second example of a clock gene, *frq*, as well as the first direct experimental proof that the core of the Oscillator was built around a transcriptional translational negative feedback loop (TTFL). Proof that FRQ was a clock component provided the basis for understanding how light resets the clock, and this in turn provided the generally accepted understanding for how light resets all animal and fungal clocks. Experiments probing the mechanism of light resetting led to the first identification of a heterodimeric transcriptional activator as the positive element in a circadian feedback loop, and to the general description of the fungal/animal clock as a single step TTFL. The common means through which DNA damage impacts the Oscillator in fungi and animals was first described in Neurospora. Lastly, the systematic study of Output was pioneered in Neurospora, providing the vocabulary and conceptual framework for understanding how Output works in all cells. This model system has contributed to the current appreciation of the role of Intrinsic Disorder in clock proteins and to the documentation of the essential roles of protein post-translational modification, as distinct from turnover, in building a circadian clock.

Keywords

WCC; FRQ; Input; Oscillator; Output

Introduction

Model Systems and Evolution

Every life scientist understands the basics of evolution and the concept of the Tree of Life, that after life originated, new life forms evolved through changes in genetic material that were passed to offspring. Species are genetically independent and arise due to genetic isolation, from physical separation or from other factors that no longer allow interbreeding,

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and these processes eventually gave rise to the incredible spectrum of living organisms seen on the planet today.

A corollary of the Tree of Life, of course, is that evolution is history dependent: Evolution doesn't act on a clean slate but instead acts on the existing palette of characteristics to make changes that allow the cell/organism to better adapt to its surroundings. Because of this, organisms that are related via a common ancestor share a common genetic and molecular history, and the molecular bases of common traits in one organism can be understood by studying the molecular basis of the trait in another related organism. This is the basis of using model systems to understand biological phenomena. Some organisms have less complex genomes, may be simpler to culture, and are more tractable to study but exhibit the same biology: to understand the physics of flight, one does not need to study a Boeing 787 because the design principles are clearly present, used, and arguably more accessible in the Wright brother's biplane.

Model systems are not meant to recapitulate all aspects of **a** phenomenon, but to distill a phenomenon to its basics and thereby to allow studies on less tractable systems to be correctly focused. Models systems, thus, often have utility in teaching one how to think about a problem. A classic biological example is the eukaryotic cell cycle whose molecular architecture is shared by plants, animals, and fungi and that was first dissected by using the yeasts Saccharomyces and Schizosaccharomyces, organisms very amenable to classical and molecular genetics and much simpler to grow and study that mammalian cells in culture. Like cell cycles, circadian clocks in fungi and animals provide another compelling example.

How can we know that fungal and animal clocks are related and do not just happen to work in the same way? For genes this is often easy to determine, as the minor DNA sequence changes needed to confer a modified trait do not change the overall sequence so much that it becomes unidentifiable as having arisen from a common ancestor; casein kinase 1 δ in Neurospora is 78% identical (same amino acid at the same place in the primary sequence) to casein kinase 1δ in humans. This is true for many of the proteins required to assemble fungal and animal clocks but not for all of them. If the rate of change is fast enough, or if the time span is long enough (like the billion plus years elapsed since fungi and animals diverged), sequence similarities can become obscured, so DNA or protein sequences cannot answer questions regarding evolutionary origins. Instances where the same trait evolved more than once are called convergent evolution. For instance, echolocation developed twice independently in the mammalian ear, in both bats and dolphins, where the same genetic mutation arose separately in a protein of outer hair cells in the ear that conferred enhanced high frequency sensitivity (Liu *et al.*, 2010). Here the clear history dependence of evolution is plainly seen in which the basic ear structure in mammals provided the substrate on which selective pressure to develop echolocation can act. Another case of molecular convergent evolution is the atomic structure of the catalytic sites of cysteine and serine proteases where structural constraints of the proteins and the chemistry of the active amino acids limited the number of possible structural solutions (Buller & Townsend, 2013). In both cases evolution could solve the problem (echolocation or proteolytic cutting) in only one way given other constraints. In other cases of convergent evolution quite different solutions were reached when there were fewer constraints. A great example is the biochemistry of bioluminescence

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where the ability to enzymatically produce light has evolved independently dozens of times (Wilson & Hastings, 1998). However, a tenet of co-evolution is that, absent constraints (such as ear structure in mammals or amino acid chemistries in proteases), evolutionary solutions will not be similar (as in bioluminescence).

In this context consider the molecular oscillators that underlie circadian rhythms. This problem, how to build a daily clock, has been solved by evolution at least three times with quite different solutions to the same design problem, namely, how to build a circadian timekeeper that allows anticipation of environment changes and regulation of important cellular events so they happen at the most advantageous time of day. In cyanobacteria a posttranscriptional clock comprised of just three proteins is at the core (see chapter by Golden in this volume). In higher plants an extremely complex series of inter-dependent feedback loops generates time (Hsu & Harmer, 2014; McClung, 2014). In fungi and animals a single step transcription/translation feedback loop underlies the rhythm. Among the fungi and animals with clocks, the positive arm of the feedback loop centers on a heterodimeric transcription factor comprised of two proteins interacting by PAS domains; it acts to drive expression of genes encoding proteins that depress (within the negative arm) the activity of the positive arm. As discussed more thoroughly below, not only is this molecular architecture of the circadian oscillator quite similar in fungi and animals, but similar or virtually identical proteins are involved doing similar activities at each of the steps. Given the fact that nature found (at least) two other completely distinct ways to make a circadian clock, there is little support for the notion that fungi and animals, which share the most recent common ancestor among all the Kingdoms of Life, just happened to converge on the same molecular solution - any more than there is reason to believe that animal cells happened to converge on the same way to build a cell cycle as did yeasts. Fungal and animal clocks work in basically the same way, and because fungi (or more specifically the predominant fungal model system used to study clocks, *Neurospora crassa*) are so experimentally tractable, much of what is known about animal clocks was first discovered by studying Neurospora.

What We All Wanted to Know about Circadian Oscillators

In the generation before anything was known about the molecular nature of circadian oscillators, a series of questions were carefully framed. The phenomenon was strictly defined by leaders in the field including Pittendrigh, Hastings, Aschoff, **Bünning** and others (Chovnick, 1960): Not just any rhythm could be called circadian, but instead only those that persisted under constant conditions; only those that had a period of about a day; only those that could be reset by brief interruption in the constant regimen; and importantly, only those whose period length was compensated against ambient changes in the environment, the phenomenon known as compensation. These restrictions excluded the long period growth rhythms induced in some fungi by particular nutritional conditions (e.g. Jerebzoff, 1976; Shi *et al.*, 2007), excluded cell cycles (as they are not compensated), and excluded a wide variety of other natural cycles. Interestingly however, strictly defining the circadian phenomenon allowed circadian biologists to perceive underlying similarities in seemingly disparate biologies such as photoperiodism and sun-compensated celestial navigation, both of which are now understood to be governed by the circadian system. By 1960 most, and by 1970

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nearly all biologists interested in circadian rhythms shared a common view of the questions. It was clear that a clock could be assembled at the level of a single cell, as indeed they all were in microbial systems, although it was not at all well accepted that all clocks would be based at the level of cells, as is now known. Beyond this, everyone wanted to know how the core of the clock was assembled, including the identity of the gears and cogs, the nucleic acids and proteins, and how they worked together; they also wanted to know how compensation worked. Together, answers to these two questions would describe how the Oscillator worked. Additionally, they wanted to know how clocks could be entrained by pulses of light and temperature, and in particular how it was that a simple unidirectional environmental cue such as a light pulse could be differentially interpreted to yield phase advances or delays depending on the circadian time of day when the pulse was perceived (Input). They wanted to know how a single oscillator could control all of the things in organisms that are clock-controlled (Output).

Work on Neurospora provided the first answers to questions regarding Input and Output and also to parts of the Oscillator question for clocks in fungi and animals. This includes the nature of the positive element (the heterodimer) at the core of the Oscillator, proof that transcriptional negative feedback lay at the core of the Oscillator, the mechanism of light-resetting and of temperature resetting, and the finding that clock control of gene expression is the principle means of circadian output. The details and recent advances in each of these areas have been described in a number of thorough reviews (e.g. Loros, 2008; Zhang *et al.*, 2011; Goldsmith & Bell-Pedersen, 2013; Gyongyosi & Kaldi, 2014; Sancar & Brunner, 2014; Zamborszky *et al.*, 2014; Cha *et al.*, 2015; Montenegro-Montero *et al.*, 2015; Hevia *et al.*, 2016; Dunlap & Loros, 2017; Koritala & Lee, 2017); these threads will not be duplicated here, but instead the emphasis will be on the particular examples and primary literature where work on Neurospora described, predicted, or first informed research that led to similar findings in mammalian clocks. A corollary of this emphasis is that this review necessarily focuses heavily on work done before the early 2000's. Before the late 1990s, molecular work on mammalian clocks was almost nonexistent; the first mammalian clock gene was not cloned until 1997 (Tei *et al.*, 1997), but then progress was rapid due to foundational work on the model systems Neurospora and Drosophila. These two systems had mapped out how fungal/animal clocks worked, information then translated to various mammalian cells as these systems became more tractable. While excellent work continued on Neurospora and Drosophila after 2000, many studies tended to emphasize exact details of how each system worked rather than more global findings relevant to all fungal/animal clocks. I have made no attempt to cover modeling work in Neurospora and how it may have contributed to understanding modeling work in other systems.

What We All Learned about Circadian Oscillators from Neurospora

Historical overview: By the time Jerry Feldman left Colin Pittendrigh's lab at Princeton in 1967 with a PhD and a solid knowledge of circadian clocks, the definition of rhythms was consolidated enough to imagine a search for clock mutants. This formed the basis of his work as a postdoc in Biology at Caltech which began that year. Feldman chose Neurospora as the organism in which to look for mutants as a clock had already been described by Pittendrigh and colleagues and Sargent and colleagues in this organism (Pittendrigh *et al.*,

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1959; Sargent *et al.*, 1966), and it was well adapted for genetic, and importantly biochemical studies. During Feldman's first year at Caltech Ron Konopka arrived as a first-year graduate student in Biology and in the following summer also initiated work aimed at uncovering rhythm mutants in a different system, *Drosophila melanogaster*. Mutants were identified in both systems (Feldman & Waser, 1971; Konopka & Benzer, 1971; Feldman & Hoyle, 1973) but lay fallow for some years both because the molecular techniques that would comprise the cloning and genetic engineering revolution had not yet been invented, and also because a dominant thought in the rhythms field at the time was that genetics would not be a useful approach for understanding clocks. By the end of the 1970s, however, technologies had advanced to where it was possible to imagine the cloning of clock genes. Independent work was initiated at about the same time in Drosophila (the Young lab at Rockefeller and the Hall and Rosbash labs at Brandeis) as well as in Neurospora where Jay Dunlap's postdoctoral fellowship in the Feldman lab at UC Santa Cruz included the goal of cloning *frq*. By the time Dunlap took the project with him as an assistant professor in 1984 no clock genes had yet been cloned.

Work on the Neurospora clock took two approaches. One was NIH-supported work focused on the cloning of the clock gene *frq* and the other, NSF-supported, used a novel technique called subtractive hybridization aimed at identifying genes whose expression was under the control of the clock. The NIH supported ideas were based on a long history of work in the rhythms field showing that brief inhibition of protein synthesis could reset the clock (e.g. Taylor *et al.*, 1982; Dunlap & Feldman, 1988) in a manner similar to the action of light, work that was interpreted as meaning that clock-critical proteins had to be expressed at a particular phase of the cycle; if so this temporal regulation of expression had to arise either from clock-controlled transcription or translation. The beauty of the subtractive hybridization approach was that it had the potential to identify both clock components and also genes involved in clock output, although in the event it identified only the latter.

During the 1980s clock genes were cloned, *per* in 1984 and *frq* in 1986 (reviewed in Dunlap, 2008). The first large scale unbiased screen for circadian cycling transcripts was completed in Neurospora (Loros *et al.*, 1989). The clock-controlled genes were called "*ccgs*" but *frq* was not identified among the first set of cycling transcripts, probably due to its low abundance. Kathy Siwicki in Jeff Hall's lab showed that the Per protein cycled in abundance (Siwicki *et al.*, 1988) paving the way for the description of cycling of *per* gene expression and a proposal for an autoregulatory transcriptional feedback loop (Hardin *et al.*, 1990).

So by 1990 mRNA transcripts that cycled in abundance (*ccg*s) had been identified, providing an obvious means for circadian Output and also a clue as to a mechanism that was consistent with extensive inhibitor work from the prior decade. Clock genes were known in the two basic genetic model systems for clocks, but the sequences of neither the PER nor FRQ proteins provided any clue as to their actual activities (although it did prompt an interesting tangent in which PER was temporarily thought to be a proteoglycan (Jackson *et al.*, 1986; Reddy *et al.*, 1986)). A negative feedback loop with an unknown number of steps was posited as the core of the clock, but whether this loop closed within cells, between cells, or via behavioral/developmental Output was unknown (Hardin *et al.*, 1990), where and how clock proteins acted was unknown, and literally nothing was known about Input (Figure 1).

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Answers to all of these questions, how Input works, biochemical activities associated with circadian Oscillator components, and how Output works, emerged first from study of FRQ and *ccg*s in Neurospora.

Components and Regulatory Architecture of the Oscillator

All published work on the mechanism of the circadian Oscillator through the 1980s and early 1990s was descriptive, and resulted in identification of cycling molecules (*per*, *ccgs, frq*) but only guesses as to their roles in circadian biology. Cycling of *per* and *frq* mRNAs was consistent with their participation in a feedback loop but proved nothing beyond their regulation. The actual testing of models for clocks was not feasible as this required the ability to regulate the expression of clock molecules, e.g. to hold their expression constant in time, or express them in a controlled pulsatile manner, all through a means that did not itself affect the clock; this was not possible until tools were developed to do this in Neurospora. Norman Giles and Mary Case had studied quinic acid metabolism extensively in Neurospora, and this information was adapted for circadian research. Quinic acid is an obscure alternative carbon source; the genes and proteins required for its metabolism are induced by the presence of quinic acid in the growth medium, and the transcription factors through which this induction happened were known (Giles *et al.*, 1985). Using this deep knowledge, it was possible to create hybrid DNA molecules in which the promoter from a gene that was induced by quinic acid could be used to drive expression of *frq* (Aronson *et al.*, 1994). When the inducer, quinic acid, was added to strains bearing the quinic-acid-inducible *frq* gene, the resulting constitutive expression of *frq* stopped the clock whereas controls showed that addition of quinic acid to wild type strains did not affect the clock (Aronson *et al.*, 1994). Further work showed that constant over-expression of FRQ via the inducible promoter depressed expression of *frq* from its native promoter, providing the classic textbook example of negative feedback. As the title of this paper (featured on the cover of Science) noted, this autoregulation in the clock gene *frequency* was the first experimental proof of negative feedback defining a circadian clock. It established the FRQ protein as a central component of a negative feedback loop that acted unambiguously in a feedback loop that closed within cells to regulate its own cyclic expression, expression whose cycling was essential for the clock and whose phase determined the phase of the clock (Figure 2). This was the first experimentally validated model for the mechanism of a circadian Oscillator.

Input—Knowledge that cycling *frq* expression lay at the core of the Oscillator provided the foundation for understanding the means through which light resets the clock. Sue Crosthwaite was able to show that *frq* was strongly and acutely light-induced; that is, light acted just like quinic acid induction in the hybrid strains to rapidly change the level of FRQ (Crosthwaite *et al.*, 1995). This finding, combined with the work of Aronson et al. (1994) showing the rhythm in *frq* expression provided an explanation for circadian entrainment by light and moreover directly answered the conundrum mentioned above, how a simple unidirectional cue such as a light pulse could be differentially interpreted to yield a phase advance or a delay depending on the circadian time of day when the pulse was perceived. As predicted from clock theory, light-intensity and duration showed reciprocity so that the combination of the two resulted in *frq* induction and phase resetting that increased linearly

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with light exposure. Translation of protein, not simply expression of mRNA, was required for the effect.

This is best explained with a figure (Figure 3). An added outcome from the explanation of light-resetting developed in Crosthwaite et al. was the ability to predict the mechanism of light-entrainment for the Drosophila clock. In Neurospora, *frq* cycles with a peak in the early subjective day and so light-induction of *frq* works to push the phase of the clock after light-exposure to daytime (Figure 3). In Drosophila, however, *per* expression peaks at night rather than in the day so light-induction of *per* could not be the mechanism for resetting in flies. Instead we predicted (Crosthwaite *et al*., 1995) that light must lead to the turnover of *per* or its protein product Per. This prediction was later shown to be the case (Hunter-Ensor *et al*., 1996). All animals and fungi use one of the methods predicted by Crosthwaite et al., either induction of the gene encoding a negative element of the feedback loop as seen in Neurospora and mammals, or turnover of a negative element protein as in insects.

In early 1997 Hitoshi Okamura and colleagues (Tei *et al*., 1997) identified a sequence homolog of the *per* gene in mice, and showed that it cycled in abundance with a phase exactly like that of *frq* with a peak in the daytime. It was then a small step to posit that resetting of this mammalian clock worked just like resetting in Neurospora, via acute light-induction of *mPer1* in the SCN, the anatomical center controlling the mouse behavioral rhythm. This was confirmed by collaborative work (Shigeyoshi *et al*., 1997) showing that, as in Neurospora, light-intensity and duration showed reciprocity so that the combination of the two resulted in *mPer1* induction and in phase resetting that increased linearly with light exposure; light induced *mPer1* at night but not in the day. These data provided the first molecular explanation for mammalian light-resetting, showing that at the cellular level it worked in the same way as in Neurospora and conceptually the same but mechanistically differently in insects.

Temperature changes are, in addition to light, the principal zeitgebers for all circadian clocks, so the mechanism underlying temperature resetting is of broad interest. A clue to the mechanism for temperature resetting came in observations that FRQ protein exists in two forms, one 100 amino acids longer than the other, and that the ratio of the two forms changes with temperature (Liu *et al*., 1997). Looking more closely, it became clear that the absolute amount of FRQ in the cell increases as a function of temperature, despite the fact that *frq* mRNA levels are not sensitive to temperature. This provided a pleasing model for temperature resetting (Figure 4). Unlike the case with light where resetting is always due to gene induction or protein turnover, a variety of mechanisms have been described for temperature resetting ranging from heat shock responses to temperature-sensitive neuronal activity or gene circuits (Buhr *et al*., 2010; Kidd *et al*., 2015; Barber & Sehgal, 2018), so while the Neurospora model was the first, and may have provided some framework for thinking about resetting mechanisms, unlike light-resetting it did not describe the universe of possibilities.

Although light and temperature changes are the most universal zeitgebers, complex clocks also receive input from a variety of factors within cells to facilitate responses to the environment. We now know one of these is DNA damage with the key to understanding the

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mechanism of this response coming from Neurospora. A Neurospora clock mutant, *period*-4, originally identified in the Feldman lab (Gardner & Feldman, 1981) was characterized by a short period length and altered temperature compensation, and cloning of the gene revealed it encoded the DNA-damage response kinase Checkpoint Kinase 2 (CHK2) (Pregueiro *et al.*, 2006). Normally CHK2 is quiescent unless it is activated by DNA damage, but interestingly, the defining mutation in *period*-4 did not eliminate its function but instead made it constantly active, even in the absence of DNA damage. Among the substrates of CHK2(PRD-4) in Neurospora was the FRQ protein. Prequeiro *et al.* showed that DNA damage reset the clock, and that this resetting required normal CHK2(PRD-4) function. By this time in the field it was widely appreciated that the long-time delay of the circadian cycle was due in large part to phosphorylation of the negative elements such as FRQ and PER, so it could be understood that the period shortening was due to premature phosphorylation of FRQ during the cycle by CHK2(PRD-4). That is, normally CHK2(PRD-4) would only become active following DNA damage when it would act on the clock, via FRQ, to reset its phase, presumably to aid in the DNA damage response. In the mutant, because CHK2(PRD-4) was always active, FRQ was always being phosphorylated by CHK2(PRD-4), and the clock runs fast. CHK2 was also found to cycle, resulting in clock regulation of the DNA damage response (Pregueiro *et al.*, 2006). The knowledge from Neurospora that DNA damage resets the clock via CHK2 prompted similar studies in mammalian cells with strikingly identical results: mammalian cellular clocks are also reset by DNA damage when the damage activates CHK2 which in turn phosphorylates mammalian clock proteins such as PER2 (Oklejewicz *et al.*, 2008; Gamsby *et al.*, 2009).

Components and Regulatory Architecture of the Oscillator – Reprise

The knowledge that *frq* was light-induced led naturally to the question of what transcription factors were required for this induction. In Neurospora, two genes were known through mutation to be required for all light responses, *wc*-1 and *wc*-2, and we determined that, as expected, there was no light-induction of *frq* in *wc* null mutants. Unexpectedly, however, these strains were arrhythmic, a finding explained initially by the assumption that without the ability to sense light the clocks simply could not be synchronized. The *wc*-1 and *wc*-2 genes were both cloned in the mid 1990s by Giuseppe Macino and colleagues (Ballario *et al.*, 1996; Linden & Macino, 1997) and their products shown to form a heterodimeric transcription factor, the White Collar Complex or WCC. We then realized that the right circadian experiment was to see whether temperature steps, which were known to synchronize the clock but were not sensed by the WCC, could synchronize the clocks in *wc*-null strains; surprisingly they did not, and in fact *frq* levels were vanishingly low in the *wc*-null strains. Further work confirmed that the WCC had two roles: in the light it acts as a chromophore-binding photoreceptor (Froehlich *et al.*, 2002; He *et al.*, 2002), the first molecular circadian photoreceptor to be identified. In the dark it was required as a positive element to drive *frq* expression. WCC, a heterodimeric DNA-binding transcription factor whose components interact via PAS domains, is the positive element in the circadian feedback loop (Crosthwaite *et al.*, 1997). This finding provided a wealth of new information. Prior to the identification of the WCC as the positive element in the feedback loop, no biochemical activities had actually been assigned to clock molecules. Now clock proteins could be said to be DNA binding transcriptional activators, which formed a heterodimer via

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PAS domains, and that were known to be required for expression of a gene, *frq*, known to encode a protein that depressed its own expression (Crosthwaite *et al.*, 1997). It had previously been shown that the kinetics of *frq* induction and inhibition, alone, took 22 hours (Merrow *et al.*, 1997) so there was no need to invoke additional steps in the Neurospora feedback loop. Therefore, the negative feedback loop underlying rhythmicity was described by WCC driving expression of *frq, frq* mRNA being translated into FRQ protein, and FRQ protein acting to depress the activity of the WCC.

The WCC research article ushered in an exciting year in clocks. Two weeks after the publication of WCC as the positive element, the Takahashi lab published the sequence of the mouse CLOCK gene, revealing that it contained both a DNA binding domain and a PAS domain (Antoch *et al.*, 1997; King *et al.*, 1997), and moreover was a member of a family of proteins that formed heterodimers via PAS domains (Hogenesch *et al.*, 1998). In short order the other member of the heterodimer was identified as BMAL1 (MOP3) (Darlington *et al.*, 1998; Gekakis *et al.*, 1998), providing a rapid and remarkable confirmation of the role of heterodimeric PAS-domain interacting transcription factors as positive elements in the circadian feedback loop in fungi and animals. Further confirmation came just over a year following WCC and CLOCK, when genetic screens in Drosophila identified, again, two proteins (CLK and CYC) with sequences indicating that they were transcription factors with PAS domains (Allada *et al.*, 1998; Rutila *et al.*, 1998). In all, these data were sufficient to propel "circadian rhythms" into the runner-up slot for Science's "Breakthrough of the Year" in 1998 and to nucleate the basic model of the fungal and animal clock as we now know it (Figure 5). It should be noted that although the Neurospora work was first to identify a heterodimeric circadian activator, the work from mouse and Drosophila identifying CLOCK/ BMAL1 and CLK/CYC had begun years before in the same way that the molecular work identifying *frq* had begun years before *per* was cloned. But as with *frq and per*, the Neurospora work was available to provide insights into the subsequently published work in animals.

Through the next decade, work on Neurospora contributed to the developing sense of the importance of post-translational control of clock proteins. While the specific phosphorylation events are generally organism-specific, the kinases that are involved in Oscillator or Output appear to be largely conserved and include CK1 and CKII (Yang *et al.*, 2002; Yang *et al.*, 2003; He *et al.*, 2006) as well as PKA, CAMK (Huang et al., 2007), and several phosphatases (Yang *et al.*, 2004; Schafmeier *et al.*, 2008) (reviewed in Cha *et al.*, 2007; Querfurth *et al.*, 2007). As important as the kinases themselves, work on Neurospora was contemporaneous with development of the general model in which clock protein-mediated phosphorylation of positive elements is the mechanism for circadian negative feedback (He & Liu, 2005; Schafmeier *et al.*, 2005); this mechanism has recently been explicitly validated through identification and functional testing of over 100 phosphosites on WC-1 and WC-2 (Wang *et al.*, 2019).

Two final aspects of clock proteins and circadian Oscillator mechanism to which Neurospora has contributed foundational knowledge have recently emerged and are now facilitating insights into mammalian clocks. The first is the experimental validation that clock proteins such as FRQ (and PER) are "Intrinsically Disordered Proteins" or IDPs (Hurley *et al.*, 2013;

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Dunlap & Loros, 2018). Clock proteins BMAL1, CLOCK and CRY have also been reported as at least partially disordered proteins (Partch *et al.*, 2005; Czarna *et al.*, 2011; Michael *et al.*, 2017). Normally proteins assume a structure that reflects the primary sequence of their amino acids, but in IDPs there is no inherent structure; they can assume a more-or-less random coil and, importantly, post-translational modifications such as phosphorylation can induce structure and influence the ability of IDPs to interact with other proteins. In this way they are ideally suited for nucleating and controlling the formation of complexes that can change over time, just what is needed for the assembly of circadian activating and repressing complexes (e.g. Aryal *et al.*, 2017). As an aside, the knowledge that FRQ and PER are IDPs with no inherent structure also explains why there is so little amino acid sequence conservation between these clock proteins that occupy such similar roles in the negative feedback loop. What is conserved is the regulatory architecture of the loop, along with the predominant kinase (CK1) and the positive elements (PAS heterodimeric transcription factor), but not the sequence of the negative element.

A second aspect of the feedback loop that has most recently emerged is in many ways the most surprising. One of the longest standing observations in the molecular clock field is that the circadian cycles are characterized by daily cycles in the amount of clock proteins like PER and FRQ. From this observation came the inference that both daily synthesis of clock proteins AND daily turnover were essential for the clock to run. So the model was that clock proteins are made and repress the heterodimeric circadian activators; slowly they become phosphorylated, eventually become targets for degradation, and then are finally turned over which releases the heterodimer to reactivate the genes leading to their expression. And indeed there exist excellent *correlations* between clock protein period length and stability of FRQ and for PER (Ruoff *et al.*, 2005; Syed *et al.*, 2011), but of course correlation does not mean cause and effect. Proteins are degraded when they are recognized by certain proteins ("F-box proteins") that escort them to the proteasome, the cell's garbage disposal. The surprise came when the F-box protein responsible for FRQ (FWD-1) was deleted and the circadian clock still ran with a relatively normal period length and amplitude (Larrondo *et al.*, 2015); this simply cannot be the case for the old model to be true, and further work has revealed a model where the true source of the clock protein inactivation is not the turnover but the phosphorylation: When a protein like FRQ becomes sufficiently phosphorylated it simply has no activity and virtually ceases to exist as far as the clock is concerned; after that, turnover is apparently good housekeeping but is not a normal part of the cycle (Kramer, 2015).

This model runs in the face of many pre-existing models and preconceptions, as well as some well-entrenched interpretations of existing data, so it was of great interest when confirmatory data emerged from mammalian cells. In a tour-de-force, the Ueda lab mapped all phosphorylation sites on the core clock protein CRY1, mutated all of the phosphorylation sites individually, then measured the effect on period length as well as on stability (Ode *et al.*, 2017). They showed that while it is possible to influence period by changing stability, as has been shown to be the case in Neurospora, it was also possible to change period without changing stability and to change stability without changing period. That is, just as was the case in Neurospora, there was no obligate coupling between stability and period length. Building on the recent finding that many clock proteins display large regions of intrinsic

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disorder (e.g. Hurley *et al.*, 2013; Xu *et al.*, 2015), the universal observation that clock proteins in fungi and animals display slow cycles of phosphorylation across the day, and the finding that when these cycles are studied, the placement of phosphorylations is not at all random but is instead highly reproducible across the day (e.g. Baker *et al.*, 2009; Tang *et al.*, 2009; Chiu *et al.*, 2011), these mammalian data and the Neurospora FWD-1 data can be rationalized in a model where there are two types of phosphorylations (Figure 6). One type, "Clock Signaling Phosphorylations" (CSP), modify the clock proteins in manners that influence the spectrum of interacting proteins in clock relevant complexes. At the end of the day the proteins like FRQ and CRY1 are terminally modified so that they lose their ability to interact in functionally relevant ways, so in a sense they disappear from the clock even though they may still be physically present in the cell and even in complexes. These last phosphorylations then lead to further phosphorylations of a functionally distinct class, so called "Degradation Signaling Phosphorylations" (DSP); they facilitate recognition of the terminally phosphorylated clock protein by the cell's garbage disposal, the proteasome, but the kinetics of this act of cellular housekeeping isn't important for the Oscillator. If housekeeping is quick, as it is in normal cells, there will be a good correlation between period length and protein stability as is indeed observed in normal cells, but if the garbage disposal is compromised (as in a cell lacking FWD-1), then housekeeping is compromised and the correlation between period length and stability breaks down but the clock continues to run. It is imperative that some specific forms of these clock proteins cycle for clock function, as they cannot be supplied constitutively (Aronson *et al.*, 1994), but overall turnover is not necessary.

Output—Circadian Output is the most organism-specific aspect of every circadian system so in some ways it would seem the least likely to be conserved among a range of organisms that is as diverse as animals and fungi or even all organisms containing clocks. Information in cells is stored in DNA, however, and this introduces constraints on how this information can be accessed in a time-of-day-specific manner: Information has to come from DNA, through RNA and then generally into proteins. For a clock that is built upon transcription and translation it is perhaps not surprising that circadian control of the first step of this process, transcription, is the principle means that cells use, yielding a spectrum of clock-controlled genes, *ccg*s. This is the universal common denominator for the first tier of circadian Output not only in fungi where this was first described, but also in mostly all other eukaryotic clocks (Loros *et al.*, 2003).

As noted above, the first systematic screen for clock-controlled genes (*ccg*s) was carried out in Neurospora in the mid 1980s, designed both to identify genes involved in Output as well as genes whose products were a part of the central Oscillator. However, technical aspects of the screen and the available technologies caused transcripts expressed at very low levels, such as those encoding clock proteins like *frq*, to be missed. However, the initial screen did successfully identify two genes involved in Output and in naming them added the term "*ccg*" to the circadian lexicon (Loros *et al.*, 2003) (Loros *et al.*, 1989). Since this time, both in Neurospora and in a variety mammalian cells, numerous studies have utilized ever more sophisticated techniques for identifying *ccgs*, going from subtractive hybridization to differential hybridization, to counting ESTs, to microarrays, and finally to RNA-seq in the

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most recent literature. Sophisticated algorithms are now available for identifying *ccg*s from time series data and the field has settled on "best practices" for carrying out such studies (Hughes *et al.*, 2017). Although the number of *ccg*s in any cell is a function of the growth conditions as well as the confidence level applied to the data, as a gross generalization roughly 10%–20% of genes in any eukaryotic cell can be considered as rhythmically present; in Neurospora growing in liquid culture, expression of up to roughly 40% of the genome is clock regulated (Correa *et al.*, 2003; Hurley *et al.*, 2014).

The existence of *ccg*s provides an obvious model for Output that has influenced how the field views this (Figure 7): The daily cycle of the clock results in rhythmic activity of the heterodimeric activator (e.g. WCC or CLOCK/BMAL1), so Output is achieved when this activator drives expression of genes whose products do not participate in the feedback loop. The realization that the time at which the amounts of different *ccg* mRNAs reach their peak spans the whole cycle, however, highlights an additional question: If the activity of the heterodimer peaks at a specific time of day, as indeed it must, then how is it that the spectrum of *ccg*s spans the entire day? There must be means for phase reversal and also for fine-tuning of the expression of individual genes, and the initial steps for this are likely to be where the heterodimer drives expression of first tier transcription factors (TFs) that then act on downstream genes; phase reversal could be achieved simply when the heterodimer drives expression of a repressor. This can be conceptualized into a model shown in Figure 8 based on existing RNA-seq and ChIP-seq data from Neurospora but easily generalized to any cell type. When clock-regulated TFs driven by the heterodimer in the Oscillator regulate each other as well as regulating downstream genes in a combinatorial manner, the network of regulators will form a dynamic filter for time information generated by the Oscillator such that, in the final step of Output all phases of the cycle are represented. While the specifics of Output will certainly be different in each cell type, the overall design principles may be quite similar, and harkening to a statement made at the beginning, the value of model systems is to aid in learning how to conceptualize questions in more complicated systems.

While circadian control of the transcriptome plays a central role in control of Output it is not the sole route for time information and work on Neurospora has contributed to understanding these alternative routes. A significant body of work from the Bell-Pedersen lab has developed the theme of circadian control of MAPK signaling in Output control (e.g. Vitalini *et al.*, 2007; Bennett *et al.*, 2013; Goldsmith *et al.*, 2018) (reviewed in Goldsmith & Bell-Pedersen, 2013) that has foreshadowed similar work in mammalian cells, and work on Neurospora has contributed to understanding how the circadian system gates mitosis and the cell cycle (Hong *et al.*, 2014). Most recently a thread of research is emerging that documents a major role for post-transcriptional control in Output. Using novel software algorithms developed for dealing with large time series proteomics data sets (Crowell *et al.*, 2018), Hurley *et al.* (Hurley *et al.*, 2018) have sampled a 25-fold larger portion of the circadian proteome than previously possible and shown that over 40% of clock-controlled proteins arise from non-rhythmic RNAs, thus documenting a significant role for circadian post-transcriptional control. This regulation is due in large part to circadian post-translational control of eukaryotic elongation factor 2 (eEF-2) (Caster *et al.*, 2016), a major factor governing mRNA translation in eukaryotes.

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Conclusions

Neurospora has provided a number of insights that have advanced our understanding of animal clocks, and has solidified other models. Work on the molecular bases of the Oscillator began in Neurospora before any clock genes were cloned and provided the second example of a clock gene, *frq*, as well as the first direct experimental proof that the core of the Oscillator was built around a transcriptional negative feedback loop. Proof that FRQ was a clock component provided the basis for understanding how light resets the clock, and this in turn informed the first and still correct general understanding for how light resets all animal and fungal clocks. Experiments probing the mechanism of light resetting led to the first identification of a heterodimeric transcriptional activator as the positive element in a circadian feedback loop, and to the general description of the fungal/animal clock as a TTFL surrounded by additional ancillary loops that provide stability and robustness. The common means through which DNA damage impacts the Oscillator in fungi and animals was first described in Neurospora. Lastly, the systematic study of Output was pioneered in Neurospora in work that provided the vocabulary and conceptual framework for understanding how Output works in all cells, and that continues to inform the study of Output in animal cells. Work on this model system has contributed to the current appreciation of the role of Intrinsic Disorder in clock proteins and is serving to redirect the focus on Oscillator study away from clock protein turnover, which has been shown in both animals and fungi to be a correlated but not an essential process for the normal clock, and towards post-translational modification of clock proteins.

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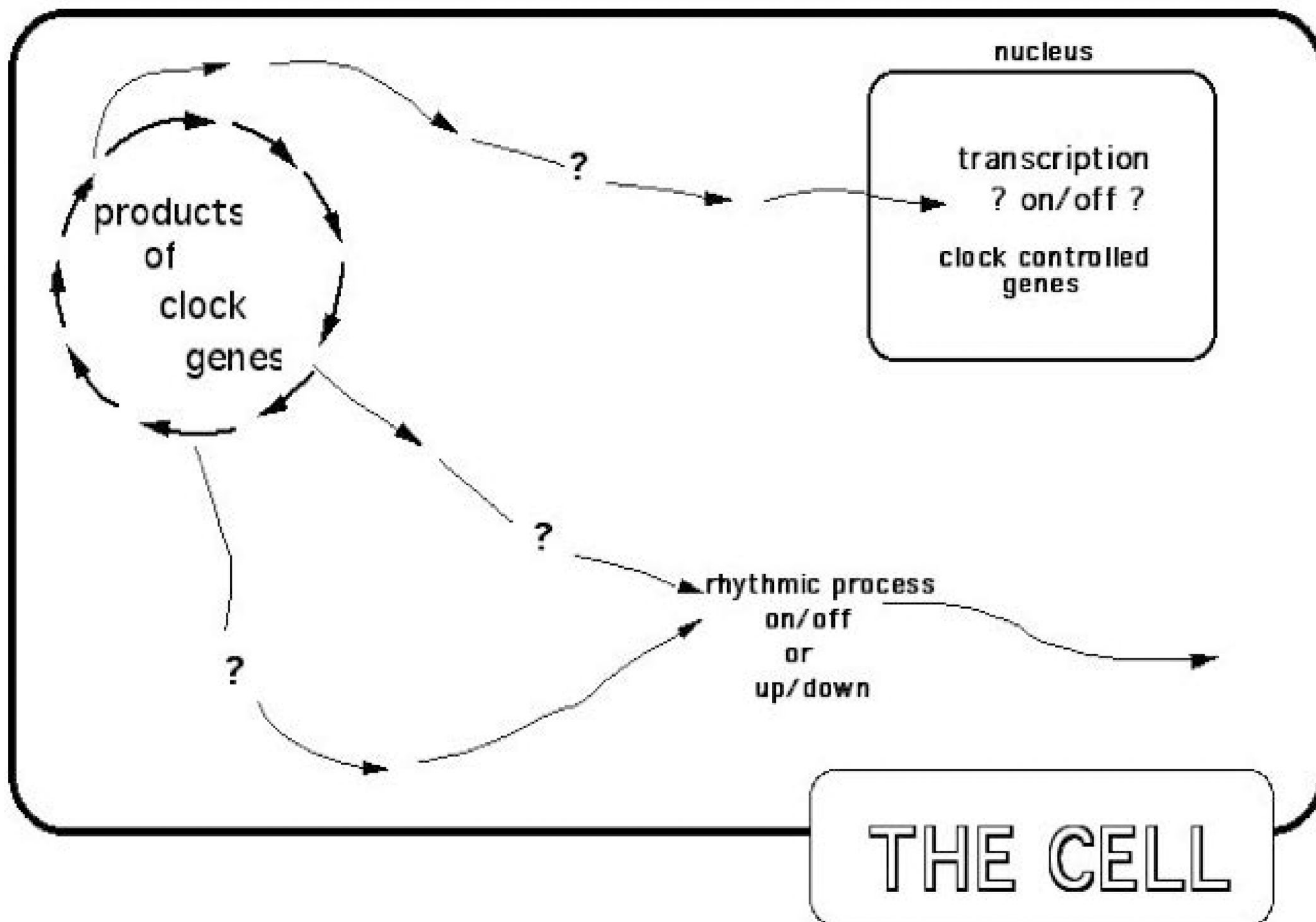

Fig. 1. The first cartoon of a molecular clock within the cell.

A simple model from 1993 depicting a hypothetically conserved molecular clock evoking a cycle of clock genes of unknown number of steps. FRQ had been shown to be a central component in a negative feedback loop required for clock function. Additionally, transcriptional activation and/or repression is known at this time to be a means of circadianly regulated output in Neurospora. (Dunlap, 1993)

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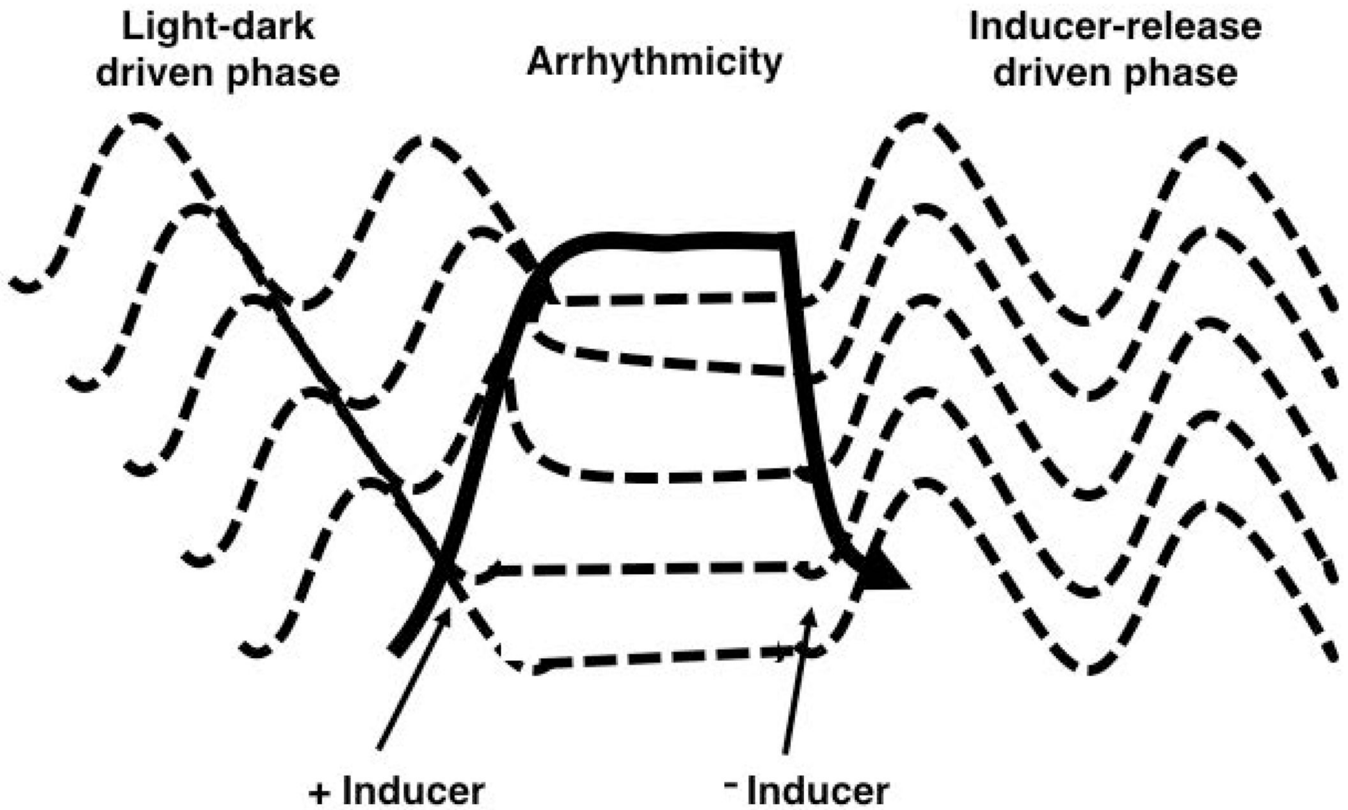

Fig. 2. Step increases and decreases of the clock gene FRQ resets the phase of the rhythm.

Shown is a schematic of results **demonstrating** auto-regulation of the Neurospora oscillator. Five sets of Neurospora cultures were transferred from light to dark every 5 hrs resulting in out of phase, staggered rhythmic cultures (dotted black lines on left). The *frq* gene was driven by an inducible promoter. Addition of inducer results in an increase in ectopic FRQ encoding transcript (solid black line). Increased FRQ represses the endogenous *frq* mRNA rhythm (dotted black lines in middle) resulting in low and constitutive *frq* mRNA. Washing the inducer out of the cultures results in re-initiation of in-phase mRNA oscillations (dotted black lines on right) beginning from the low point of the endogenous *frq* transcript cycle corresponding to subjective evening, thereby confirming that specific levels of FRQ correspond to distinct times in the clock cycle. (Aronson *et al.*, 1994)

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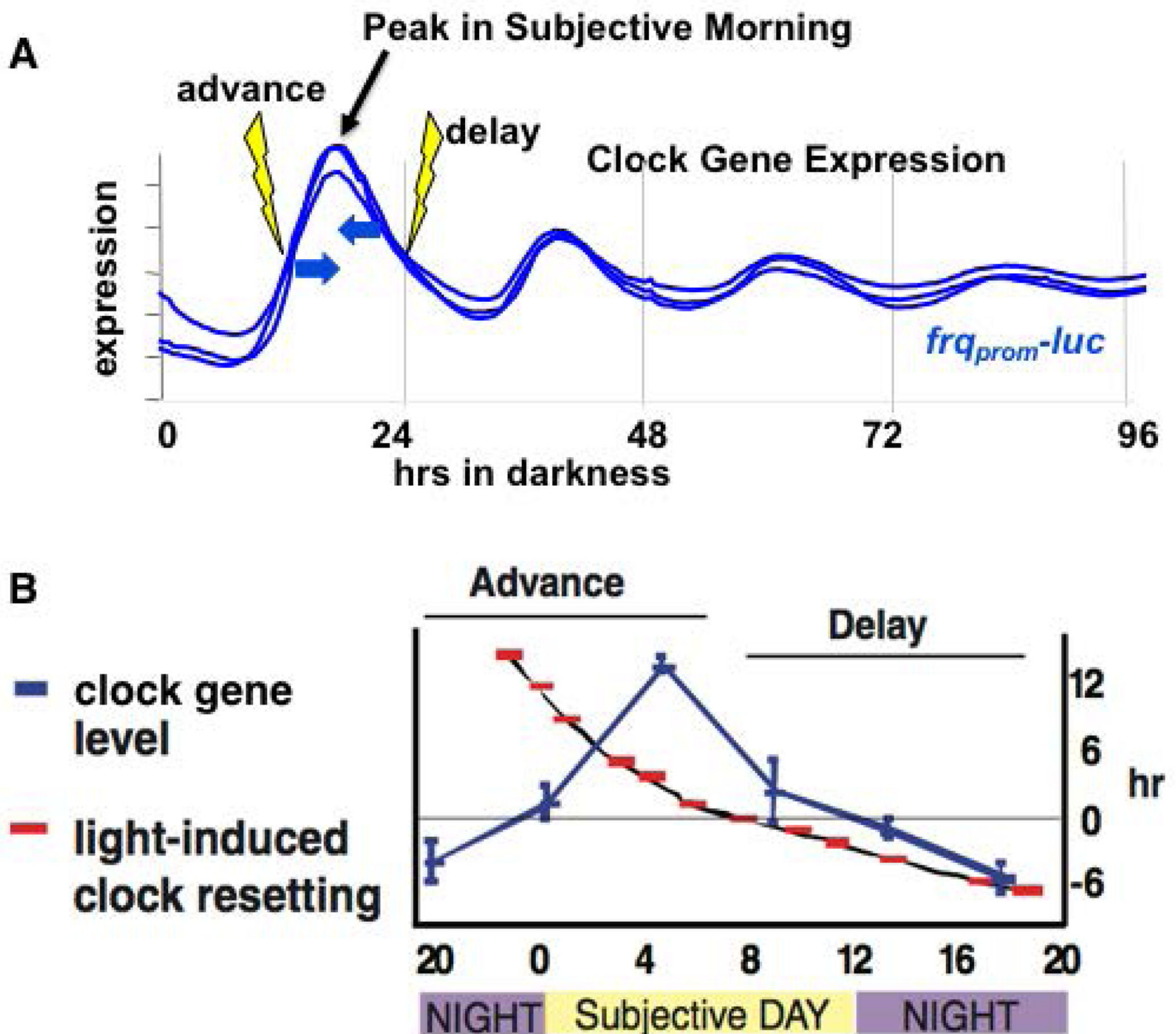

Fig. 3. How light resets fungal and animal clocks.

Light rapidly and transcriptionally induces negative element genes, *frq* or *per*, in fungi and mammals (Crosthwaite *et al.*, 1995; Shigeyoshi *et al.*, 1997). A) Blue traces show output from a luciferase reporter driven by the frq promoter clock-box element in the dark with peaks in the subjective morning. Blue arrows show the direction the peak of the mRNA will move in response to light either before or after mRNA peak. B) If the mRNAs are rising, this rapid induction will result in an advance into the day phase of the clock. If the light stimulus occurs while mRNA levels are falling, the rapid increase will push the phase of the clock backwards, resulting in a delay back to the day phase. Blue lines represent mRNA levels. Red blocks represent the phase the clock moves to after a light stimulus. (Crosthwaite *et al.*, 1995; Dunlap, 1999)

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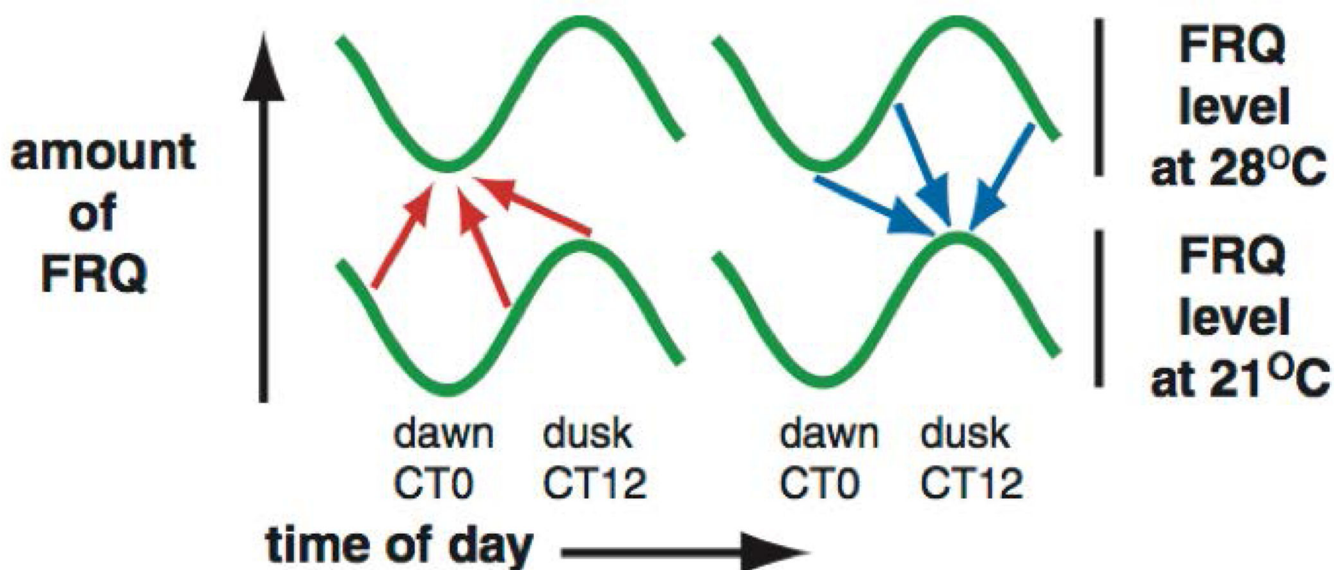

Fig. 4. How temperature resets the Neurospora clock

Cycling of FRQ protein levels over the day are represented by green lines at lower temperatures (lower curve) and higher temperatures (upper curve). Red arrows represent a temperature step up at any time of the day, always resetting the clock to the trough of FRQ protein while blue arrows represent a temperature step down, always resetting the clock to the peak of FRQ protein. With a step up in temperature all points on the low curve are below all points on the high curve so the clock is reset to the low point of FRQ, subjective late night to dawn. With a step down in temperature all points on the upper curve are above all points on the low curve so the clock is reset to the high point of FRQ, subjective late day to dusk. (Liu *et al.*, 1998; Dunlap, 1999)

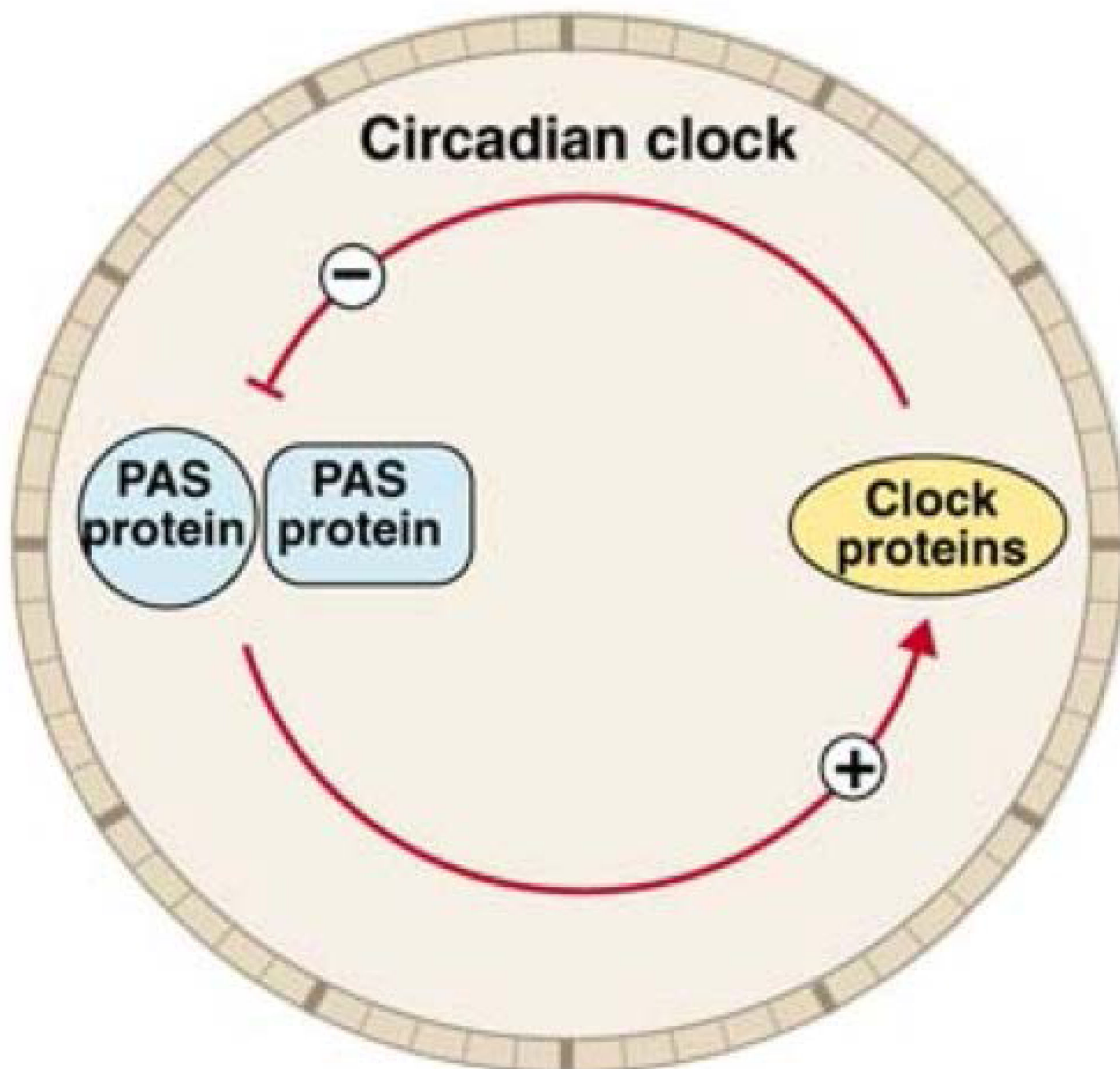

Fig. 5. A TTFL is at the core of fungal and animal clocks.

Within the cell the positive arm of the Transcription Translation Feedback Loop (TTFL) contains PAS domain containing transcription factors forming a heterodimer that drive the expression of negatively acting clock genes that feed back to directly block the activity of the transcription factors. (Dunlap, 1998)

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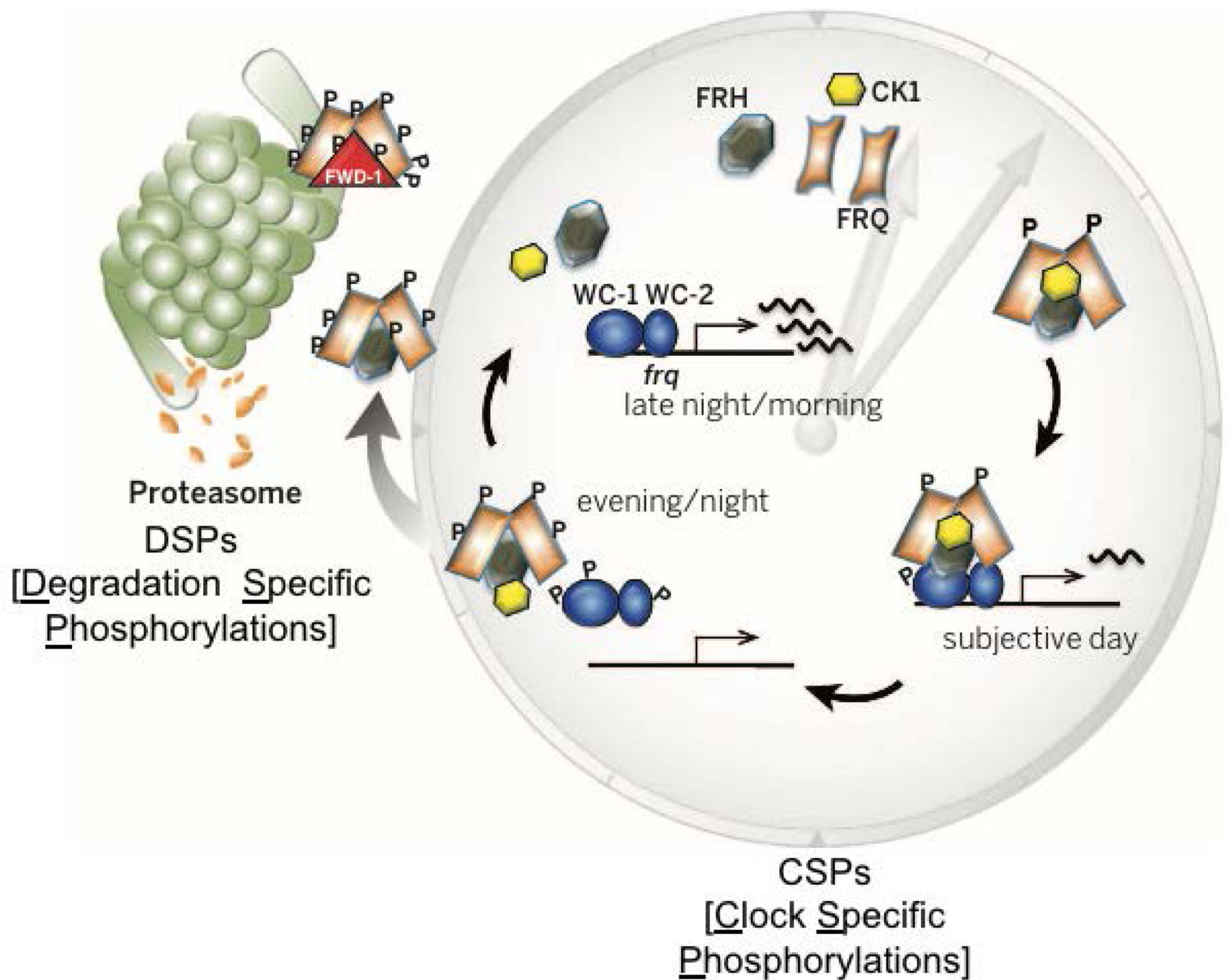

Fig. 6. Separate and specific roles for the phosphorylation of FRQ in essential negative feedback and non-essential FRQ turn over.

Events essential to the clock are shown within the shaded clock face. The WC-1/WC-2 transcription complex activates the expression of *frq*, FRQ protein complexes with FRH and CK1 and feeds back to repress the WCC activity. Phosphorylation of FRQ can reduce this interaction and determine the rate at which essential molecular events within the clock proceed. Once key phosphorylations (CSPs, Clock Specific Phosphorylations) on FRQ occur and negative feedback is complete, further phosphorylation events will not affect circadian period length but do determine FRQ turnover (DSPs, Degradation Specific Phosphorylations) by FWD-1, an E3 ubiquitin ligase, and the proteasome, shown outside the clock face. (Larrondo *et al.*, 2015)

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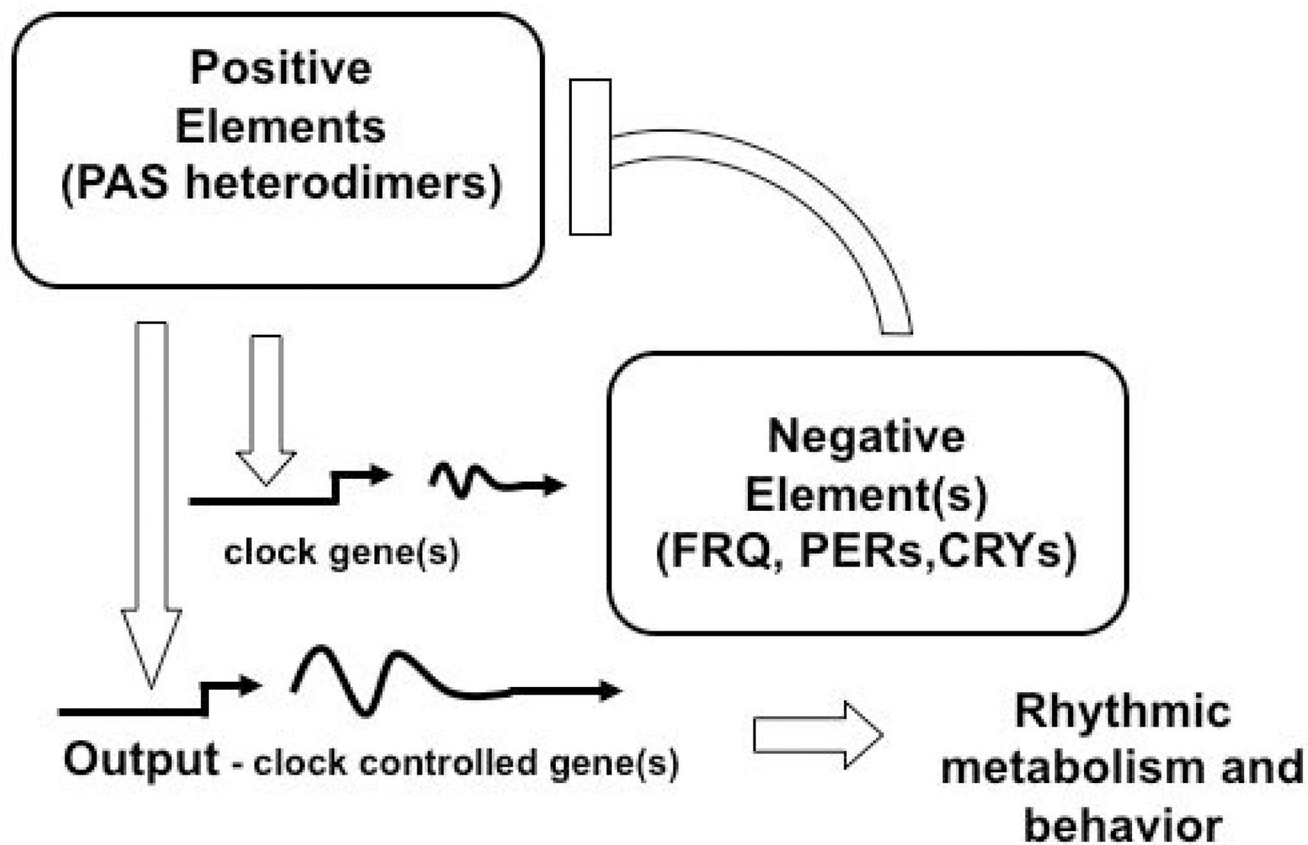

Fig. 7. Common elements in the design of fungal and animal feedback loops can directly regulate output.

The PAS domain transcription factors that drive rhythmic expression of the system dependent negative element clock proteins, FRQ, PERs and CRYs, also drive rhythmic expression of clock-controlled genes (ccgs). Cycling expression of ccg's results in rhythmic metabolism and behavior at the cell, tissue and organismal level. (adapted from Dunlap, 1999)

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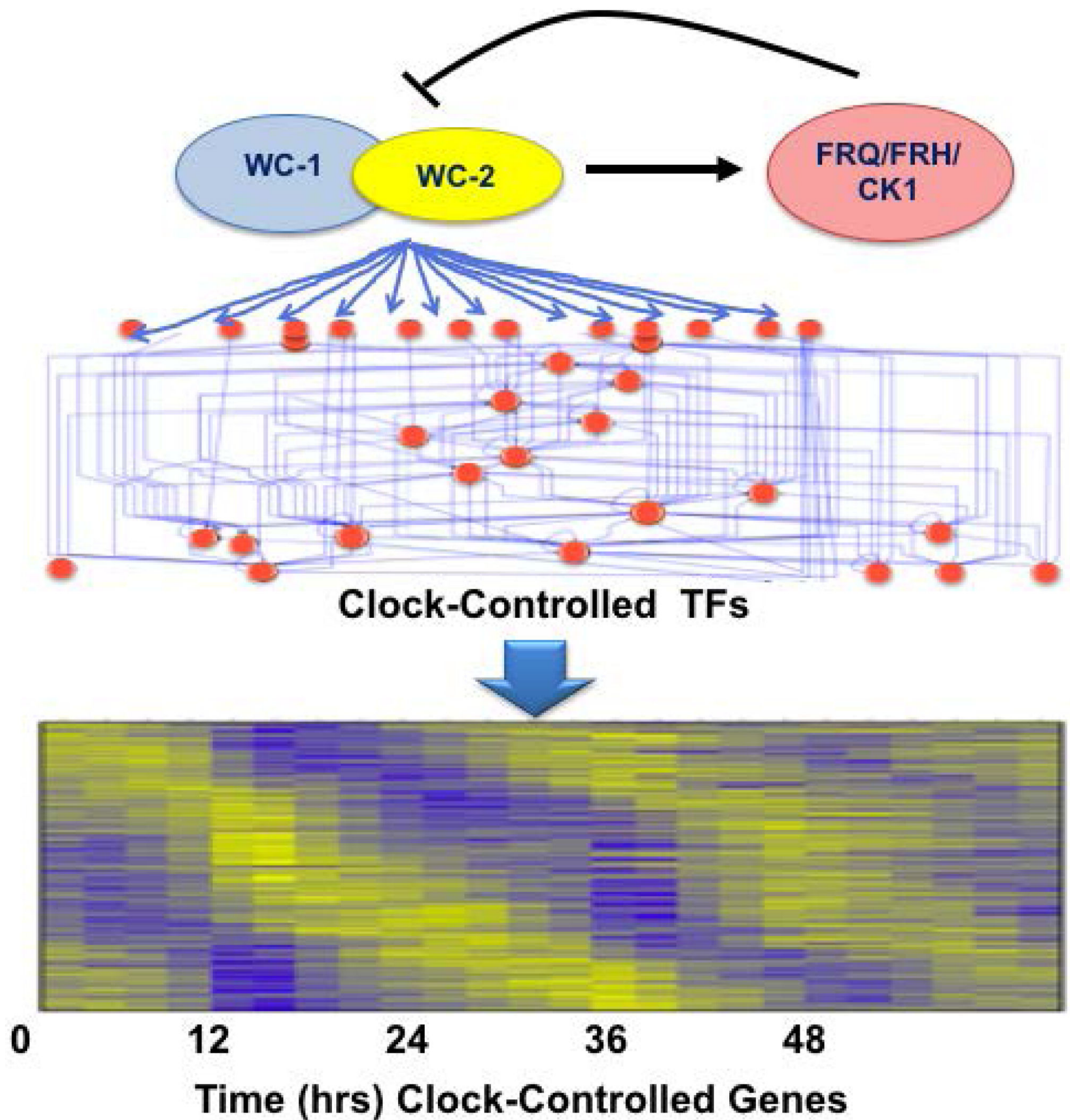

Fig. 8. Combinatorial effects of multiple rhythmic TFs result in groups of genes having independent and specific phases in Neurospora.

Top: A cartoon showing the WCC and FRQ core molecular oscillator feedback loop. Middle: The WCC directly and rhythmically activates several genes in addition to *frq*. The top tiers of the cascade are enriched in clock-controlled regulators i.e. transcription factors (TFs) and DNA-binding proteins, shown as red balls. These clock-controlled regulators in turn regulate other genes, shown as blue interconnecting lines, including more regulators in a several tiered cascade over time. In addition, many of the regulators are acutely controlled by light and/or are autoregulatory, leading to complex combinatorial regulation of gene

expression. Bottom: RNA Seq results showing hundreds of rhythmic genes with peaks of gene expression at multiple phases around the circadian cycle. (Hurley *et al.*, 2014)

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