

Redox and Metabolic Oscillations in the Clockwork

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Abstract Daily (circadian) clocks have evolved to coordinate behaviour and physiology around the 24-h day. Most models of the eukaryotic circadian oscillator have focused principally on transcription/translation feedback loop (TTFL) mechanisms, with accessory cytosolic loops that connect them to cellular physiology. Recent work, however, questions the absolute necessity of transcription-based oscillators for circadian rhythmicity. The recent discovery of reduction-oxidation cycles of peroxiredoxin proteins, which persist even in the absence of transcription, have prompted a reappraisal of current clock models in disparate organisms. A novel mechanism based on metabolic cycles may underlie circadian transcriptional and cytosolic rhythms, making it difficult to know where one oscillation ends and the other begins.

Introduction

Daily biological clocks provide living organisms with temporal organisation over a 24-h timescale. Organisms from bacteria to humans have evolved these rhythms to adapt their physiology to the solar cycle and anticipate the availability of resources (e.g., food and light; Bass 2012). Despite their presence in evolutionarily disparate organisms, the molecules underlying the clockwork seemed to be different in these organisms. This finding has given rise to the identification of “clock genes” that oscillate with 24-h periods but that are not related in their DNA or protein sequences, except in some cases when comparing fruit flies and mammals. This has led to the notion of the divergent evolution of different clock circuits in various model organisms, the only link being the way in which the components are joined together in a negative feedback loop topology (Rosbash 2009; Fig. 1).

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P. Sassone-Corsi, Y. Christen (eds.), *A Time for Metabolism and Hormones*, Research and Perspectives in Endocrine Interactions, DOI 10.1007/978-3-319-27069-2_6

Model Organism

Cyanobacteria	Fungus	Plants	Fly	Mouse
				
<i>KaiA</i> <i>KaiB</i> <i>KaiC</i>	<i>FRQ</i> <i>WC-1</i> <i>WC-2</i>	<i>TOC-1</i> <i>CCA-1</i>	<i>Per</i> <i>Tim</i> <i>Clock</i> <i>Cycle</i>	<i>Per</i> <i>Cry</i> <i>Clock</i> <i>Bmal1</i>

“ Clock Genes”

Fig. 1 The dominant model organisms used for the study of circadian rhythms are shown. Below are lists of the more important clock genes that are/were thought to mediate negative feedback loops in these organisms. At the DNA or protein sequence levels, there is no evolutionary conservation beyond some in fly and mammals

Challenging Transcriptional Models of the Clockwork

An increasing number of studies, both in higher and lower organisms, have questioned the necessity of a functional transcriptional oscillator for cellular rhythmicity. Circadian transcription is stochastic in mammalian cells (Suter et al. 2011); therefore, one would expect that perturbation of transcription during cell division would result in phase variability, which is not seen. In line with this finding, global inhibition of transcription with actinomycin D and α -amanitin has revealed the robustness of circadian oscillators to such severe perturbations, and single cells exhibit bioluminescence rhythms even when the transcription rate is reduced by ~70 % (Dibner et al. 2009).

Perhaps more importantly, studies showing that constitutive expression, or deletion, of “clock genes” does not abolish circadian rhythms call into question the importance of transcription in current clock models. In flies, expression of both *per* and *tim* under the control of a constitutive promoter can affect circadian rhythms. However, ~50 % of the flies still exhibited robust behavioral rhythms (Yang and Sehgal 2001). Similarly, fungi can exhibit conidiation (spore formation) rhythms in the absence of central components of their feedback loop [involving the *frequency (frq)* gene] (Lakin-Thomas 2006). Moreover, in some organisms, the dominant mechanism regulating circadian rhythms seems to be post-transcriptional, as exemplified by the circadian control of translation of luciferin binding protein (LBP) in the unicellular alga *Gonyaulax polyedra* (Morse et al. 1989; Mittag et al. 1994).

In mammals, the situation is difficult to dissect since circadian genes often have multiple homologues; therefore, double-mutant animals are generally needed to observe a behavioral phenotype. *Bmal1* was thought to be the only exception to this, with its suppression leading to clear behavioural arrhythmicity (Ko et al. 2006). Constitutive brain-specific expression of *Bmal1* in knock-out animals is, however, able to restore behavioral rhythmicity, questioning the necessity of rhythmic *Bmal1* transcription (McDearmon et al. 2006). In addition, brain-specific knockout of *Bmal1* expression produces gross pathology, with a striking abundance of activated microglia in the brains of mice, which gets progressively worse over the first 6 months of life. This finding makes it extremely difficult to dissociate the effects of BMAL1 as a generically important transcription factor from those specifically related to the malfunctioning of a biological clock (Musiek et al. 2013).

Even more importantly, imaging of suprachiasmatic nucleus (SCN) slices from arrhythmic *Bmal1*^{-/-} and *Cry1*^{-/-}*Cry2*^{-/-} animals with bioluminescence reporters revealed the persistence of low amplitude rhythms in individual neurons (Ko et al. 2010; Maywood et al. 2011). As recently shown, it is likely that developmental effects underlie the apparent arrhythmicity that is observed when adult animals are assayed, as is the case in most experimental paradigms (Ono et al. 2013).

There is thus considerable evidence that current transcription-translation feedback loops cannot account for the multiple lines of experimental evidence that have revealed circadian oscillations in the presence of inactivated feedback loops or indeed in their absence.

Non-transcriptional Clock Mechanisms

The experimental anomalies highlighted above suggest that other mechanisms are required to fully explain the molecular basis of circadian timekeeping. It is indeed worth underscoring that transcriptional mechanisms were regarded as only one of the several possibilities that were investigated before the discovery of “clock genes” (Edmunds 1988).

An instructive outlier in clock research is the macroscopic unicellular alga *Acetabularia*, which can maintain self-sustained circadian rhythms in photosynthetic activity when its nucleus is removed by cutting off its nucleus-containing rhizoid process (Sweeney and Haxo 1961). Intriguingly, its nucleus is able to dictate the phase of oscillation but is dispensable for entrainment and phase shifting (Schweiger et al. 1964). Moreover, inhibition of transcription with Actinomycin D did not suppress rhythms in either nucleated or enucleated *Acetabularia* cells, although the former surprisingly lost rhythmicity after 2 weeks under these conditions (Mergenhagen and Schweiger 1975). Similarly, platelets were used to show that glutathione exhibited circadian oscillations relying on de novo synthesis of this important cellular reductant (Radha et al. 1985), again in the absence of a nucleus. These examples point to the fact that current circadian models cannot explain issues

raised almost 40 years ago, in some cases, suggesting the existence of non-transcriptional rhythms.

How can we reconcile these seemingly opposite views? One way is to view transcription and translation in the current models as having limited roles in setting the pace of the oscillator and to note that they are needed to maintain the levels of clock proteins and to control circadian output functions. Accordingly, post-translational modifications of known clock proteins could be the fundamental oscillator, but the transcriptional oscillator would be important for robustness and could amplify post-translational oscillations. In fact, such a model exists in cyanobacteria, in which the master transcriptional regulator KaiC is part of its post-translational oscillator.

An alternative point of view is that circadian timekeeping might have evolved more than one clock in the cell to meet the requirements of precision, robustness and stability. In this case, the known transcriptional oscillator would be coupled to a post-translational oscillator. Post-translational modifications are an integral feature of the current transcription-translation feedback models, but a definitive post-translational oscillator has not yet been identified in eukaryotic species. The recent discovery of oxidation cycles in peroxiredoxin proteins (PRDXs) offers a new window on non-transcriptional rhythms in higher organisms (O'Neill and Reddy 2011; O'Neill et al. 2011; Edgar et al. 2012; Olmedo et al. 2012). More importantly, this finding immediately suggests a common phylogenetic origin for circadian timekeeping mechanisms in virtually all species relying on oxygen for energy metabolism (Edgar et al. 2012).

PRDX Rhythms

PRDXs are an antioxidant protein family involved in hydrogen peroxide metabolism and signalling (Hall et al. 2009). Their catalytic mechanism involves the oxidation of a catalytic cysteine residue in the enzymes' active site to sulfenic acid (Cys-SOH), which then forms a disulfide bond with another non-catalytic (and so-called 'resolving') cysteine residue. The thioredoxin system usually completes the cycle by reducing this disulfide bond while oxidising a molecule of NADPH. This catalytic loop has rapid turnover and allows the maintenance of low levels of intracellular hydrogen peroxide.

So-called 'typical 2-Cys PRDXs,' a subclass of PRDXs whose basic functional unit is a homodimer in which catalytic and resolving cysteine residues belong to different molecules of PRDX, are the main players implicated in circadian cycles. These can undergo further oxidation of their catalytic cysteine to sulfinic and sulfonic acid forms (Cys-SO_{2/3}H). The 'over-oxidised' Cys-SO₂H residues can be slowly recycled through adenosine triphosphate (ATP)-dependent reduction by sulfiredoxin (Rhee et al. 2007), whereas further oxidation to Cys-SO₃H (termed 'hyper-oxidation') is thought to be irreversible.

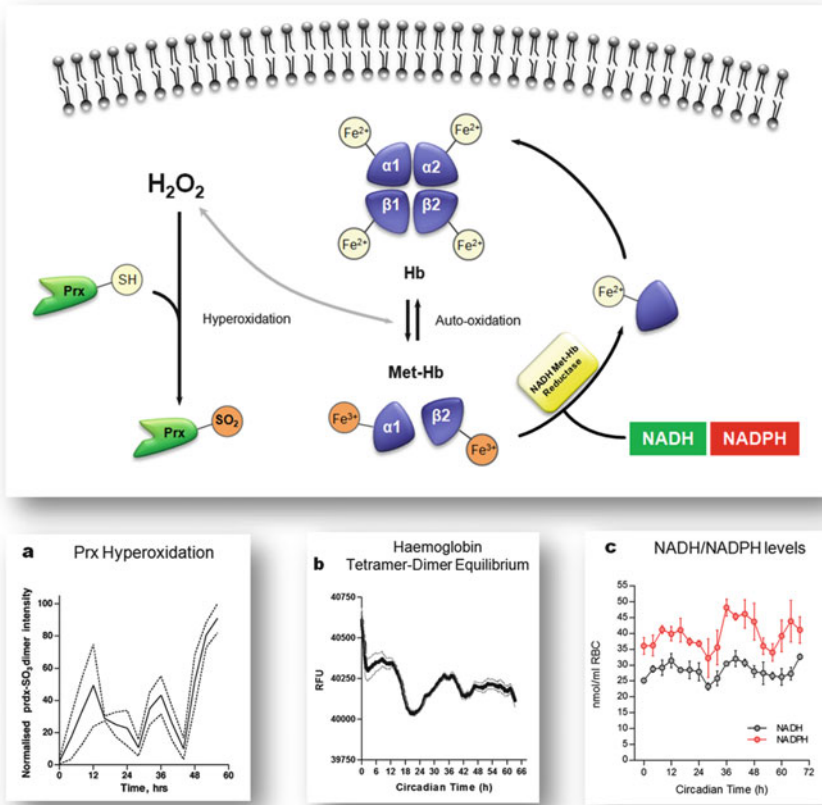


Fig. 2 A range of circadian oscillations in human red blood cells. **(a)** Oxidation of PRDXs occurs on a circadian basis in cells maintained in constant conditions (in the absence of external temporal cues) for at least 3 consecutive days. **(b)** Dynamic changes in the equilibrium of haemoglobin forms (tetramer vs. dimer states) oscillate according to a 24-h rhythm in vitro. **(c)** Oscillation of the key cellular reductants, NADH and NADPH, in red blood cells

Human red blood cells do not undergo transcription since they lack nuclei in their mature form. In these unique cells, PRDXs exhibit circadian accumulation of their dimeric over-oxidised form (PRDX-SO₂H) over several days (O’Neill and Reddy 2011). Such rhythms fulfil all criteria for circadian rhythms: (1) persistence in constant conditions; (2) the ability to be entrained (via temperature cycles in this case); and (3) temperature compensation (the clock does not run faster in higher temperatures). In addition, these redox rhythms are accompanied by oscillations in haemoglobin oxidation and metabolic variables, including NADH and NADPH (Fig. 2). Similar results have also recently been found in mouse red blood cells (Cho et al. 2014).

Rhythms similar to these are also present in the unicellular alga *Ostreococcus tauri*, even when transcription is inhibited by prolonged darkness (O'Neill et al. 2011); they are autophototrophic, requiring light for synthesis of most cellular substrates including RNA. Moreover, the deep phylogenetic conservation of PRDX redox rhythms extends to include fungal, plant, bacterial and even archaeal species. Critically, such rhythms are not dependent on previously identified clock genes, since mutants lacking circadian components maintain redox oscillations, albeit slightly phase-shifted (Edgar et al. 2012).

The phylogenetic conservation of PRDX rhythms suggests that primordial redox oscillators probably evolved following the Great Oxidation Event 2.5 billion years ago. At this time, photosynthetic bacteria are thought to have acquired the ability to produce oxygen from water, which caused a dramatic rise in Earth's atmospheric oxygen. Rhythmic production of oxygen and reactive oxygen species (ROS) by sunlight may therefore have been a critical driving force in the co-evolution of clock mechanisms and ROS removal systems that could anticipate, and thus resonate with, externally driven redox cycles (Bass 2012; Edgar et al. 2012; Fig. 3).

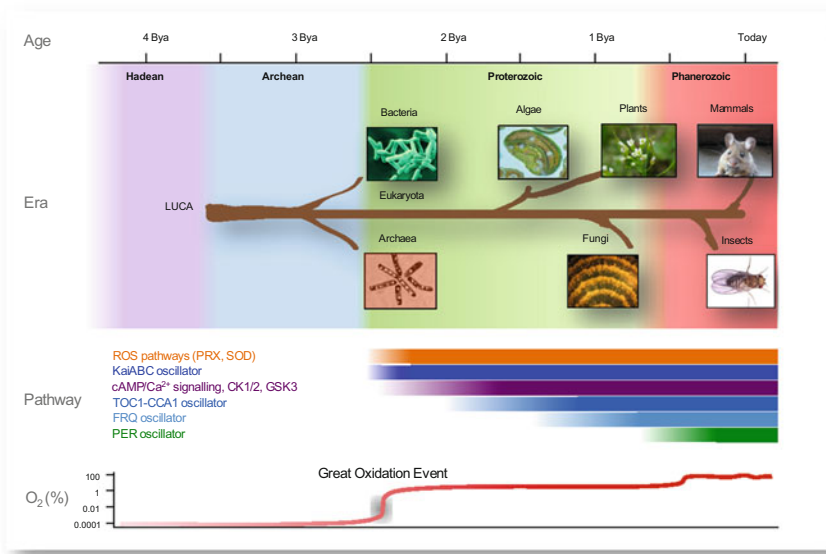


Fig. 3 Phylogenetic origins of circadian oscillatory systems. A timeline is shown at the *top* of the schematic, with the geological era illustrated. A schematic phylogenetic tree shows the origins of each organism studied, stemming from the last universal common ancestor (LUCA). The putative epoch over which each oscillator system has existed is illustrated by the labeled bars. CK1/2, casein kinase 1 or 2; GSK3, glycogen synthase kinase 3; SOD, superoxide dismutase (Adapted from Edgar et al. 2012)

Redox and Metabolic Clocks in Eukaryotes

There is clearly an interplay between circadian and metabolic cycles, and there is good evidence of reciprocal effects that disruption of one cycle has on the other at physiological and molecular levels (Bass 2012; Rey and Reddy 2013). High-fat diet, for example, lengthens the behavioral period of rhythms in mice and changes the expression pattern of clock genes (Kohsaka et al. 2007). Conversely, healthy patients subjected to 3 weeks of circadian disruption exhibit pre-diabetic symptoms (Buxton et al. 2012). The growing evidence suggesting that circadian rhythms are fundamentally metabolic requires that currently understood transcriptional oscillations are tightly coupled to metabolic cycles. This hypothesis is strongly supported by the numerous examples of accessory loops embedding the circadian transcriptional clock within cellular metabolism (Fig. 4).

An accessory loop involving $NAD^+/NADH$ is likely to play an important role in connecting cytosolic and compartment-specific redox states to transcriptional clock components such as PER2 (Asher et al. 2008) and CLOCK/BMAL1 (Rutter et al. 2001; Nakahata et al. 2008; Asher et al. 2010; Yoshii et al. 2013). In addition, other redox-sensitive mechanisms have been identified in the clockwork and, in particular, the heme-sensing transcriptional regulators (Dioum et al. 2002; Yin et al. 2007; Gupta et al. 2011).

Even in early molecular studies of the circadian clock, before “clock genes” had been discovered in any model organism, rhythms in redox had been reported. For example, in plants, $NADP^+:NADPH$ ratio exhibited circadian cycles in seedlings kept in constant darkness (Wagner and Frosch 1974). Several studies in rodents

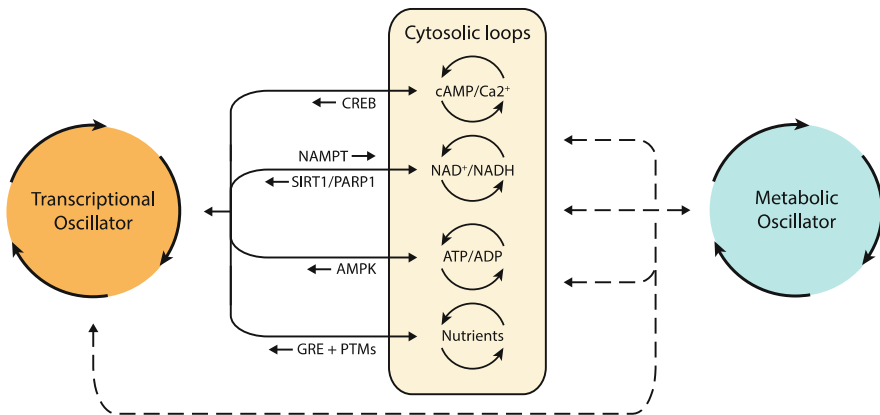


Fig. 4 Links between transcriptional, cytosolic, and metabolic cycles. Cytosolic processes are thought to be part of the transcription/translation feedback loop (TTFL). The latter are involved mainly in redox and energy metabolism and form accessory loops that are controlled by the TTFL oscillator and, in turn, feed back to it. Abbreviations: *CREB* cAMP response element-binding protein, *GRE* glucose response element, *PARP* poly(ADP-ribose) polymerase, *PTMs* posttranslational modifications (Adapted from Reddy and Rey 2014)

showed that redox parameters, including the glutathione redox ratio, were diurnally regulated in the liver, although it is possible that these oscillations might have been partially driven by food intake (Isaacs and Binkley 1977a, b; Robinson et al. 1981; Kaminsky et al. 1984; Belanger et al. 1991). Nevertheless, human platelets kept in vitro showed circadian rhythms in glutathione content (Radha et al. 1985), suggesting that feeding cycles might internally resonate with these cell-autonomous biochemical rhythms.

The hypothesis that metabolic cycles might be a fundamental mechanism underlying biological clocks has been proposed based on both theoretical and experimental observations (Roenneberg and Merrow 1999). Potential evidence for this hypothesis in mammals has come from the McKnight and Sagami groups, who have shown that BMAL1/CLOCK DNA-binding activity can be modulated in vitro by the redox poise of NAD(P)⁺/NAD(P)H coenzymes (Rutter et al. 2001; Yoshii et al. 2013). In addition, the action of BMAL1/CLOCK on the NAD⁺-producing enzymes lactate dehydrogenase (*Ldh*) and *Nampt* could potentially feed back onto intracellular redox balance (Rutter et al. 2001; Nakahata et al. 2009; Ramsey et al. 2009). These results still require in vivo confirmation, given the relatively high concentration (millimolar range) of the coenzymes used in in vitro assays previously (Rutter et al. 2001; Yoshii et al. 2013). The recent discovery of PRDX oscillations in non-transcriptional systems, however, offers supportive evidence that redox cycles can function as circadian oscillators in their own right.

It is evident that, in organisms in which metabolic oscillations have been found but transcription-translation feedback loops have not, as in the worm *Caenorhabditis elegans*, insights into metabolic oscillatory mechanisms may be easier to come by. It is thus possible that metabolic oscillations could drive PRDX oscillations in the absence of known transcriptional feedback oscillators (Olmedo et al. 2012). So-called accessory loops, including NAD⁺/NADH and NADP⁺/NADPH cycles, are potential candidates for self-sustained metabolic oscillators, but further studies of their oscillatory properties in clock mutant backgrounds will be of great interest to identify bona fide components of metabolic oscillators. However, this assumes that deletion of important circadian-relevant transcription factors itself does not lead to abhorrent redox changes in cells and tissues, as is the case in *Bmal1*^{-/-} animals (Kondratov et al. 2006), which could compromise redox oscillations indirectly.

Conclusion

Metabolic non-transcriptional cycles clearly interlock with transcriptional processes in the circadian system. The peroxiredoxin system could be part of an uncharacterised metabolic oscillator, given its broad phylogenetic conservation and its slow kinetics, which is compatible with 24-h rhythmicity. Establishing the molecular links between fundamental cellular redox metabolism and transcriptional components of the clockwork remains an exciting challenge in the field.

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