

TIME ZONES: A COMPARATIVE GENETICS OF CIRCADIAN CLOCKS

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The circadian clock is a widespread cellular mechanism that underlies diverse rhythmic functions in organisms from bacteria and fungi, to plants and animals. Intense genetic analysis during recent years has uncovered many of the components and molecular mechanisms comprising these clocks. Although autoregulatory genetic networks are a consistent feature in the design of all clocks, the weight of evidence favours their independent evolutionary origins in different kingdoms.

Most of us zigzag between sleep and wakefulness with clock-like regularity. Barring jet lag, the phase of this rhythmic behaviour is also locked to the day–night cycle: for adults that are 20–50 years old, sleep onset tends to follow nightfall by about 4–5 hours with spontaneous awakenings usually occurring 1–2 hours after sunrise. For a given adult under controlled conditions, precision of the rhythm is often much greater, with sleep onsets and offsets occurring within minutes of that predicted over many cycles¹.

There are, however, important exceptions, and some unusual patterns of human sleep have attracted a great deal of interest because of evidence for heritability. Dominant traits that are associated with advanced phase sleep have been recognized in several families. Affected family members begin sleep about an hour after sunset and are wide awake by 04.00 in the morning. In January of this year, **familial advanced sleep phase syndrome** (FASPS) was linked to a single gene mutation on chromosome 2 in one large kindred². The altered gene is period 2 (*PER2*) and the mutation blocks a phosphorylation site thought to be the substrate of casein kinase 1 ϵ . This work is the first to deduce the genetic basis of a sleep disorder in humans. More importantly, FASPS research proved that familiar genetic circuitry controls normal patterns of rhythmic behaviour in humans. In fact, the activities of orthologous genes in some animals are so well known that a mechanistic interpretation of this human syndrome has already been proposed² (see below).

What role has genetics had in shaping our ideas about rhythmic behaviour? A long history of experimentation has shown that patterns of sleep and wakefulness are determined by the action of endogenous, 24-h (circadian) timers³. However, the biochemical identities of such clocks remained a formidable puzzle in all forms of life until the arrival of a genetic focus — “as mysterious as gravity before Newton”, reads one popular chronicle⁴. Beginning ~30 years ago, genetic screens precipitated a list of possible clock factors — an abstract map of chromosomal sites that might offer clues about a mechanism. As these became targets for gene isolation, biochemistry and cell biology, physical clockworks began to surface in several laboratories.

In this review, we examine the genes and proteins that make up the divergent circadian clocks of four kingdoms. In every case, an intracellular molecular oscillator has been found, but the specific strategies for building clocks show important variations. In fact, the clock genes of plants, animals, fungi and bacteria might have recorded several independent evolutionary attempts to measure time.

Blueprints from the fly

The gene now associated with FASPS, period 2, was first identified as a constituent of the circadian clock in *Drosophila*. The earliest genetic screens for mutations that affect circadian rhythmicity were focused on this insect⁵, and on *Neurospora*⁶ and *Chlamydomonas*⁷. Although the promising clock genetics of *Chlamydomonas* received no further attention, interest

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Table 1 | ***Drosophila* circadian rhythm genes**

Gene name	Properties	References
<i>period</i> (<i>per</i>)	Mutations alter rhythmicity (arrhythmic, long- and short-period alleles). RNA and protein cycle. Physically associates with TIM. Negative regulator of CLK and CYC.	5,8,9,114,115
<i>timeless</i> (<i>tim</i>)	Mutations alter rhythmicity (arrhythmic, long- and short-period alleles). RNA and protein cycle. Physically associates with PER. Stabilizes PER. Negative regulator of CLK and CYC.	106,116,117
<i>double-time</i> (<i>dbt</i>)	Mutations alter rhythmicity (arrhythmic, long- and short-period alleles). Constitutively expressed. Protein kinase (CK1 ϵ). Physically associates with PER and PER/TIM complexes. Promotes phosphorylation and degradation of TIM-free PER in the cytoplasm and nucleus.	24,118
<i>Clock</i> (<i>Clk</i>)	Mutations alter rhythmicity. RNA and protein cycle. Physically associates with CYC. Binds to E-boxes in and promotes transcription of <i>per</i> , <i>tim</i> and <i>vri</i> .	26,27
<i>cycle</i> (<i>cyc</i>)	Mutations alter rhythmicity. Constitutively expressed. Physically associates with CLK. Binds to E-boxes in and promotes transcription of <i>per</i> , <i>tim</i> and <i>vri</i> .	27,119
<i>vri</i> (<i>vri</i>)	Mutations alter rhythmicity. RNA and protein cycle. bZip transcription factor. High, constitutive expression of VRI represses accumulation of <i>per</i> and <i>tim</i> RNA.	31
<i>shaggy</i> (<i>sgg</i>)	Mutations alter rhythmicity. Constitutively expressed. Protein kinase (GSK-3, glycogen synthase kinase-3). Promotes phosphorylation of TIM and nuclear translocation of PER/TIM complex.	12
<i>cryptochrome</i> (<i>cry</i>)	Mutations do not change rhythmicity, but produce defects in photoentrainment. RNA cycles. Circadian photoreceptor. Promotes light-dependent degradation of TIM. Might be required for function of some peripheral clocks.	120, 121

in the fly and fungal clockworks was rekindled in the mid-to-late 1980s with the molecular cloning of Ron Konopka and Seymour Benzer's *period* locus^{8,9} and Jerry Feldman's *frequency*¹⁰ (see below). Work in *Drosophila* has now identified seven genes that, together with *period*, compose a light-responsive molecular clock (TABLE 1). For *Drosophila*, it seems that most of the key genes have been identified — new mutant alleles tend to fall into previously defined complementation groups. Several alternative approaches to genetic screening have probably eliminated the gaps that might come from missed genes that affect viability, are redundant, or are represented by only recessive mutations (reviewed in REFS 11,12). However, the list of genes that could make a subtle contribution to clock function could be much longer¹³.

Genetics has not only been a tool for gene discovery in *Drosophila*, but also identified interactions among clock genes and their products that indicate how molecular oscillations are produced (TABLE 1). Period (PER) and its partner protein, Timeless (TIM), accumulate rhythmically and regulate their own expression (FIG. 1). Two transcription factors, Clock (CLK) and Cycle (CYC) coordinately activate the *per* and *tim* genes through shared sequences (E-boxes) in their promoters (TABLE 1). Nuclear PER suppresses this activity. Self-sustaining oscillations are generated by separating intervals of activation and suppression. This is accomplished through a series of stepwise delays, for which the casein kinase 1 ϵ orthologue Double-time (DBT) is essential (for detailed reviews of the fly mechanism, see REFS 11,14,15).

DBT is constitutively produced and physically associates with both PER and PER/TIM complexes¹⁶ (FIG. 1a). Because DBT will phosphorylate and destabilize PER, but only when PER is free of TIM, the kinase retards accumulation of PER, while cytoplasmic TIM accrues. High concentrations of TIM promote formation of stable DBT/PER/TIM complexes that can enter the nucleus. Nuclear DBT/PER/TIM complexes are converted into DBT/PER complexes over a period of ~8–10 h in wild-type flies. The conversion is associated with progressive repression of *per* and *tim* transcription, decreased accumulation and nuclear translocation of new DBT/PER/TIM complexes, and increased PER phosphorylation^{16,17}. Nuclear PER phosphorylation is dependent on DBT function, and results in PER degradation and termination/reinitiation of the molecular cycle (FIG. 1a).

Refinement of the basic mechanism. In the fly, this skeletal mechanism is refined by the action of three additional genes, *cryptochrome* (*cry*), *shaggy* (*sgg*) and *vri* (*vri*) (TABLE 1). The first of these genes, *cry*, allows the period and phase of the clock to adjust to changing environmental cycles of light and dark. The fly CRY protein functions as a clock-specific photoreceptor^{18,19}. On exposure to light, CRY is thought to physically associate with TIM to promote rapid TIM degradation through a proteasome-dependent pathway^{18,20}. A transient loss of cytoplasmic TIM by this mechanism will produce phase delays in the molecular oscillator, whereas phase advances would be generated by light-induced nuclear TIM degradation¹¹.

localization indicated that PER (and presumably TIM) might not be transferred to the nucleus progressively. Rather, prolonged perinuclear accumulation was observed for PER in brain cells that control behavioural rhythms, followed by a shift to more rapid nuclear transport²². TIM phosphorylation precedes nuclear PER phosphorylation by several hours, with high levels of TIM (but not PER) phosphorylation detected as PER/TIM complexes begin to move to the nucleus^{17,23,24}. Shaggy seems to regulate the timing of nuclear transfer by promoting TIM phosphorylation. The timing of PER/TIM nuclear localization can be advanced by elevating Shaggy activity, and the period of *Drosophila*'s rhythms can be lengthened or shortened by, respectively, decreasing or increasing the rates of Shaggy-dependent TIM phosphorylation¹² (FIG. 1b).

Shaggy is best known for its contributions to animal development through the Wnt signal transduction pathway²⁵. Phosphorylation and degradation of a key transcription factor, **Armadillo** (which encodes a β -catenin homologue), is regulated by glycogen synthase kinase-3 (GSK-3, the kinase homologous to Shaggy). In turn, Shaggy can be inactivated by the function of another phosphoprotein, **Dishevelled**. We introduce this developmental pathway because Dishevelled has recently been established as a target for casein kinase 1 ϵ regulation: casein kinase 1 ϵ phosphorylates Dishevelled and promotes accumulation of β -catenin (reviewed in REF. 25). So, a positive (casein kinase 1 ϵ) and a negative (Shaggy/GSK-3) regulator of Wnt signalling seem to contribute a system of similarly balanced controls to PER/TIM nuclear translocation in the fly clock.

A second autoregulatory path in the oscillator controls the cycling levels of CLK (FIG. 1c). Like *per* and *tim*, *Clk* RNA is produced with a circadian rhythm. However, the *Clk* RNA and protein levels fall as *per* and *tim* RNA levels rise^{26–29} (FIG. 1d). Levels of *Clk* transcription are high in mutants that lack CLK protein, so it has been suggested that CLK somehow suppresses its own transcription. PER (or a PER/TIM complex) blocks this autoregulation^{28,30} (FIG. 1c). An attractive feature of this fusion of autoregulatory networks is that *per* and *tim* repression could be established in two sequential and synergetic stages (FIG. 1d; see also REFS 29,30 and discussion of *vri*, below). During the day, CLK protein levels fall, which might cause *per* and *tim* transcription to stall in the early evening as observed. Subsequent accumulation of nuclear PER/TIM complexes could supply a second layer of regulation that involves the direct suppression of residual CLK proteins, and prolonged inhibition of newly formed CLK/CYC complexes as *Clk* RNA and protein levels begin to rise again. Elimination of TIM and PER in the early morning would allow re-emergence of active CLK to stimulate the next round of *per* and *tim* expression.

Another pathway of autoregulation has been recognized in the fly clock through studies of the bZIP transcription factor VRI (FIG. 1c). *vri* expression cycles with the same phase as *per* and *tim*, and E-boxes akin to those associated with *per* and *tim*, are found in the *vri* promoter³¹. As expected, these are positively regulated by

CLK/CYC, and negatively regulated by PER/TIM. Constitutive, high-level expression of *vri* suppresses *per* and *tim* transcription, and eliminates circadian behavioural rhythms. These, and other findings, showed an essential role for VRI in the *Drosophila* clock³¹. Although the direct targets of cycling VRI regulation are still unknown, work in mice has indicated that E4BP4, the most closely related bZIP transcription factor in mammalian genomes, is also rhythmically produced *in vivo* and suppresses *Per* expression *in vitro* by binding the *Per* promoter³². Another possibility in flies is that VRI indirectly influences *per* and *tim*, by suppressing CLK/CYC activity before the arrival of the PER and TIM proteins, or VRI might contribute to the autoregulatory loop that generates cycling *Clk* expression (FIG. 1c). Whatever the specific mechanism in *Drosophila*, high levels of VRI should join with low levels of CLK to generate the initial downturn in *per*, *tim* and *vri* expression that is observed in the early evening (FIG. 1d).

Cells that contain this light-ENTRAINABLE molecular oscillator have been detected throughout the fly, ranging from tissues that compose the gut and excretory system, to sensory cells of the antennae, legs and wings (reviewed in REFS 11,15). Although the specific rhythmic outputs of most of these cells are unknown, we are beginning to understand how rhythmic patterns of subordinate gene activity are linked to the molecular clock. Many genes outside the central clockworks bind CLK/CYC and are switched on by these transcription factors. Genes outside the oscillator are therefore repressed by cycles of nuclear PER^{11,15}.

A related plan from the vertebrates

Orthologues of the *Drosophila* clock genes have been found and variably characterized in zebrafish, *Xenopus*, several species of birds, hamsters, mice and humans. Three functional *Per* paralogues have been found in mammals (TABLE 2). The activities of these cycle robustly, as in the fly (reviewed in REFS 14,33–35). In cultured human cell lines, casein kinase 1 ϵ regulates the phosphorylation of PER proteins, which affects both their stability and subcellular location, as in the fly^{36–38}. The connection between PER and casein kinase 1 ϵ in mammals is best understood through studies of the circadian mutation *tau*, which shortens the locomotor activity rhythms of hamsters to ~20 h (REF. 39; TABLE 2). The mutant hamsters produce casein kinase 1 ϵ with a single amino-acid substitution that is defective for PER phosphorylation *in vitro*⁴⁰. In normal day–night cycles, *tau* hamsters are early risers, showing a syndrome closely resembling humans affected by FASPS³⁹. In the FASPS kindred studied by Toh *et al.*², a serine-to-glycine substitution in the human PER2 protein suppressed phosphorylation of casein kinase 1 ϵ targets in PER2 *in vitro*. So, the similar behavioural phenotypes of FASPS humans and *tau* hamsters seem to stem from a common molecular problem — aberrant formation of hypophosphorylated PER (FIG. 2).

As in the fly, *Per* transcription in mammals seems to be activated by orthologues of CLK and CYC (TABLE 2; CLOCK and BMAL1 (official human symbol **ARNTL**),

ENTRAIN

To establish the phase of a rhythm by providing an environmental signal, such as a light or temperature cycle, or a biological signal, such as a hormone pulse.

Table 2 | **Mammalian circadian rhythm genes**

Gene name	Properties	References
<i>Period1 (Per1)</i> <i>Period2 (Per2)</i> <i>Period3 (Per3)</i>	Mutations alter rhythmicity in rodents and humans (<i>PER2</i> altered in human sleep disorder, FASPS). RNA and proteins cycle. Physical associations with CRY and among PER proteins. Positive regulator of <i>Bmal1</i> .	2,122–124,126
<i>Timeless (Tim)</i>	Circadian function not established. Homozygous null is embryonic lethal. Closest relative in <i>Drosophila</i> is <i>timeout</i> . Constitutively expressed. Physically associates with CRY. Modest negative regulator of <i>Per</i> and <i>Cry</i> transcription <i>in vitro</i> .	44,125,127–129
<i>Casein kinase 1ε (CK1ε)</i>	Mutations alter rhythmicity in rodents. Affected by hamster mutation <i>tau</i> . Protein kinase, orthologue of <i>Drosophila dbt</i> . Physically associates with and phosphorylates PER. Affects PER stability and nuclear localization.	2,36–40
<i>Circadian locomotor output cycles kaput (Clock)</i>	Mutations alter rhythmicity in rodents. Constitutively expressed. Physically associates with BMAL1. Binds to E-boxes in and promotes transcription of <i>Per</i> and <i>Cry</i> .	41,130
<i>Bmal1/Mop3</i>	Mutations alter rhythmicity in rodents. Rhythmically expressed. Orthologue of <i>Drosophila cycle</i> . Physically associates with CLOCK. Binds to E-boxes in and promotes transcription of <i>Per</i> and <i>Cry</i> .	131–133
<i>Cryptochrome1 (Cry1)</i> <i>Cryptochrome 2 (Cry2)</i>	Mutations alter rhythmicity in rodents. RNA cycles. Physically associates with and stabilizes PER. Negative regulator of <i>Per</i> and <i>Cry</i> transcription.	49–51,134–136

respectively). In fact, one of these genes, *Clock*, was initially recognized by genetic screens and molecular cloning in the mouse, not the fly⁴¹. The *Clock* mutant mouse produces long-period locomotor-activity rhythms that degrade into arrhythmicity. The mutant also reduces *Per* expression⁴². A genetic knockout of the partner protein of CLOCK, BMAL1, eliminates behavioural rhythmicity and blocks *Per* expression, proving a role for these transcription factors in mammalian clocks⁴³. Mammalian CLOCK/BMAL1 complexes also serve as key links to intracellular output pathways in vertebrates as in the fly^{14,33}.

All three PER proteins can weakly suppress CLOCK/BMAL1-dependent *Per* transcription in cultured mammalian cells, and genetic knockouts of two of the three paralogues (*Per1* and *Per2*) strongly affect behavioural rhythms in mice^{44–46}. These results would seem to support a role very similar to that seen for PER in *Drosophila*. However, none of the work done in the mouse has indicated that any of the PER proteins is a particularly strong autorepressor *in vivo*. Instead, the primary role of the best studied of these proteins, PER2 (now of FASPS fame), might be the activation of *Bmal1 (Arntl)*⁴⁷ (FIG. 2). A switch in the mode of regulation of CLOCK/BMAL1 complexes in comparing flies and mice is linked to a potentially diminishing role of PER as a negative autoregulator in mammals. Recall that in the fly this complex rises and falls in abundance owing to the oscillating transcription of *Clk (cycle)* does not cycle). In the fly, PER (or a PER/TIM complex) functions as a rhythmic, positive regulator of *Clk* (FIG. 1c). In the mouse, the job of promoting rhythmicity again falls to PER (PER2), but the target has become the partner to *Clk, Bmal1*⁴⁷. So, it is *Clk* that is the constitutively expressed partner in the mouse (FIG. 2). To summarize, if a common theme is to be found with regard to PER function, it is that PER is a positive reg-

ulator of rhythmically produced CLOCK/BMAL1 complexes in both mice and flies.

If a negative autoregulatory role has been dropped for one or more of the mammalian PER proteins, do we have something in mammals that turns the *Per* genes off — a factor in mammals that would stand in for the additional negative autoregulatory role of PER in *Drosophila*? The answer seems to be yes, with another revision. This job has fallen to the mammalian CRYPTOCHROMES. Genetic deficiency for two CRY (cryptochrome) proteins in the mouse (*CRY1* and *CRY2*) stops the clock, but in these mutants *Per* is upregulated^{48–50} (TABLE 2). In contrast to the PER proteins, cultured-cell studies indicate a powerful effect of each mammalian CRY protein as a repressor of *Per* transcription⁵¹. So, whereas CRY was a photoreceptor in flies, it seems to have been recruited to work more deeply in the clock in mammals (FIG. 2).

This conclusion is bolstered by another CRY function in the mouse — CRY, rather than TIM, seems to physically associate with PER to influence its stability (presumably susceptibility to casein kinase 1ε), and to regulate nuclear translocation of PER/CRY complexes^{33,47,51} (FIG. 2). Once in the nucleus, CRY downregulates *Per* and *Cry* expression. So, what is the role of TIM in mammals? Null mutants are inviable, which precludes behavioural analysis, *Tim* transcription fails to cycle and the TIM protein is constitutively nuclear. *Tim* is also more closely related to *timeout*, a *tim* paralogue of unknown function in the fly, than it is to *tim* itself. Nevertheless, mouse TIM is found to be associated with cryptochromes in the suprachiasmatic nucleus (SCN), a structure in the mammalian brain that is responsible for organizing behavioural rhythms (reviewed in REFS 11,33). And just as we are about to completely divorce the functions of cryptochromes in mammals and flies, Selby *et al.*⁵² provide evidence for

CRYPTOCHROME
A novel photoreceptor, discovered in plants and subsequently found in animals, that is thought to have evolved from photolyase (light-activated DNA-repair protein). Cryptochromes bind flavin and pterin, and promote redox reactions upon absorbing light.

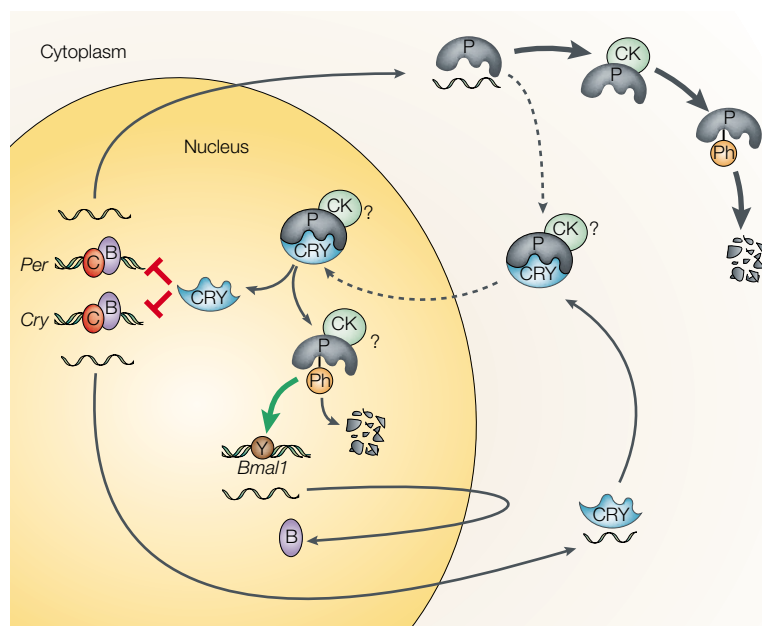


Figure 2 | Regulatory interactions in the mammalian clock. Casein kinase 1 ϵ (CK1 ϵ) seems to affect PER stability and nuclear localization. For some PER proteins, phosphorylation by CK1 ϵ could mask a PER nuclear localization signal³⁶. Formation of PER/CRY complexes promotes nuclear translocation in cultured cells and *in vivo*. Nuclear PER2 and CRY might have different targets in the nucleus: PER2 upregulates expression of *Bmal1*, whereas CRY1 and CRY2 negatively regulate *Per* and *Cry* transcription. This separation of functions is illustrated schematically, but CRY and PER might act in association with each other and/or other proteins in the nucleus. B, BMAL1; CK, casein kinase 1 ϵ ; C, CLOCK; CRY, CRY1/2; P, PER1–3; Y, PER2 might enhance the activity of a positive regulator or suppress a negative *Bmal1* regulator; Ph, phosphate. Dashed arrows, possible delays.

CRY involvement in mammalian photoreception. It also seems that in certain clock-containing cells of *Drosophila* (antennal neurons), CRY function is required autonomously for sustained molecular rhythmicity⁵³. Therefore, CRY might be a component of the clock if you test the right cells in a fly.

As in *Drosophila*, many tissues outside the mammalian nervous system show robust molecular cycles of clock gene expression. Even immortalized fibroblasts can produce circadian rhythms of clock gene expression⁵⁴. It has been reported that these cell-culture clocks depend on regulatory interactions that are indistinguishable from those discovered in the intact animal⁵⁵. *In situ*, the peripheral oscillations can be fiercely independent of the brain. For example, there is ordinarily a constant phase difference of a few hours between *Per* RNA cycles in the SCN and the liver. Light hitting the retina entrains the SCN, which presumably generates a diffusible signal that affects the phase of the hepatocyte rhythms. However, oscillating liver cells will take their phasing cues from feeding time rather than from the SCN if conflicted^{56,57}. The preference for a phase related to feeding is beginning to be understood in terms of the many target genes that are subject to circadian regulation in the liver. Out of ~50 cycling targets, most were found to encode factors that are involved in the processing and detoxification of nutrients⁵⁸. Although there is no evidence for photoreception associated with molecular oscillators in mammalian liver, kidney, muscle and lung, and behavioural

rhythmicity seems to be entrained through ocular photoreception in mammals, zebrafish have a remarkable array of peripheral oscillators, at least some of which are independently photoreceptive (for example, heart and kidney)⁵⁹. In this respect, this translucent fish is therefore reminiscent of the fly.

The alternative strategy of *Neurospora*

Soon after the description of activity rhythm mutations at the *period* locus in *Drosophila*, mutations of the *Neurospora* clock gene *frequency* (*frq*) were reported (TABLE 3). The formation of spore-containing conidia occurs with a circadian rhythm in this fungus, and the new mutations shortened or lengthened the period of the rhythm, or caused arrhythmic sporulation. Our current understanding of the fungal clock comes mostly from the analysis of three *Neurospora* genes — *frq*, *wc-1* and *wc-2*. *frq* was cloned first, and its mRNA levels were shown to cycle^{10,60}. Overexpression of FRQ protein represses endogenous *frq* expression, which provides the first clue that autoregulation of gene activity is also a central theme of the fungal clock⁶⁰. Although there are no extensive homologies between the proteins of metazoan clocks and those of other kingdoms, *Neurospora*'s WC-1 and WC-2 each contain PAS MOTIFS⁶¹ similar to those found in PER, CLOCK and BMAL1 (REF. 62), and PAS motifs have been found in at least two clock-associated proteins of the weed *Arabidopsis* (described below). For WC-1, human BMAL1 and the plant proteins, some additional similarities are found adjacent to PAS motifs^{62,63}.

WC-1 and WC-2 also contain zinc-finger DNA-binding motifs and heterodimerize through their PAS domains to form a white-collar complex (WCC). These complexes bind the *frq* promoter and activate transcription^{62,64–67}. FRQ proteins, in turn, homodimerize and interact with WCC to inhibit *frq* transcription^{68–70} (FIG. 3). Although mRNA levels of both *wc* genes show no cycling, levels of WC-1 cycle with a phase almost opposite to that of FRQ. Mutations at the *frq* locus affect both cycling and levels of WC-1 proteins, which indicates that FRQ proteins somehow promote translation or stability of the positive element WC-1 (REF. 62). WC-2 proteins are much more abundant than WC-1 (REF. 69), so the latter protein effectively generates oscillations in functional WCC.

In contrast to the behaviour of nascent PER, TIM and CRY proteins in metazoan clocks, the newly synthesized FRQ protein almost immediately translocates to the nucleus (reviewed in REF. 71), where it binds to the WCC and inhibits its transcriptional function on the *frq* promoter (FIG. 3). As indicated above, the rising level of FRQ enhances accumulation of WC-1 protein from a static RNA pool. By late afternoon, the FRQ protein level reaches its peak. FRQ also undergoes progressive phosphorylation that seems essential for its subsequent degradation and for establishing period length, as in animal clocks (reviewed in REF. 71). Overnight, *frq* transcription is impeded and FRQ proteins are degraded. When FRQ levels are lowest, WCC, having lost its

PAS MOTIF

These motifs (PER/ARNT/SIM) are often associated with proteins that function as environmental or developmental sensors. They also promote physical associations among various transcription factors.

Table 3 | **Neurospora circadian rhythm genes**

Gene Name	Properties	References
<i>frequency (frq)</i>	Mutations alter rhythmicity (arrhythmic, long- and short-period alleles). RNA and protein cycle. Physically associates with WC-1 and WC-2. FRQ proteins homodimerize, and negatively regulate <i>frq</i> transcription. FRQ stability regulated by phosphorylation.	10,137
<i>white collar-1 (wc-1)</i>	Mutations alter rhythmicity. RNA constitutive in constant darkness, but induced by light. In the dark, WC-1 protein cycles due to positive regulation by FRQ. Physically associates with WC-2 to form the white-collar complex (WCC). WCC binds the <i>frq</i> promoter and also physically interacts with FRQ protein.	64,138
<i>white collar-2 (wc-2)</i>	Mutations alter rhythmicity. Constitutively expressed. Physically associates with WC-1 to form WCC. WCC binds the <i>frq</i> promoter and also physically interacts with FRQ protein.	64,139
<i>vivid (vvd)</i>	Not required for circadian cycles, but mutations affect phase of rhythms. RNA and protein cycle. <i>vvd</i> expression is transiently induced by light. VVD seems to suppress activity of WCC. Most important role might be in photo-entrainment of the clock.	73,140

inhibitor, reinitiates transcription from the *frq* promoter to restart the cycle (FIG. 3).

WCC is also the principal mediator of light-induced gene transcription in *Neurospora*, including light-induced phase resetting of the clock. Light somehow frees the WCC from the inhibitory action of FRQ, and WCC, in turn, produces transient elevations of *wc-1*, *wc-2*, *vivid (vvd)* (see below) and *frq* RNA levels, which ultimately reset the clock (reviewed in REF. 71). The WCC response to light could involve photoreception by the complex itself; in addition to the PAS domain, WC-1 and WC-2 also contain motifs suggestive of CHROMOPHORE binding^{64,72}.

This light-induced clock resetting is sensitive to the time of day, and this regulation seems to be affected by another protein, VVD⁷³. VVD is a novel PAS-motif-containing protein, and its expression is both clock and light regulated. VVD controls some aspects of light input and clock output^{71,74}. It is presumed to physically interact with the WCC through its PAS domain and to modulate WCC function, thereby providing a mechanism for circadian regulation of the WCC light response (FIG. 3).

The long-studied circadian sporulation phenotype is a good example of how profoundly the clock regulates aspects of growth and development of this fungus. Sporulation is a mechanism that allows fungi to become airborne, to travel long distances and to survive stress. Sporulation is accompanied by substantial changes in cell-wall/membrane composition and cellular physiology. So, it is not surprising that many clock-controlled genes identified so far in *Neurospora* are involved in general stress responses, membrane reorganization, cell metabolism and developmental signalling⁷¹. The clock in *Neurospora* is, however, more broadly active in vegetative growth and function, indicating a pervasive involvement in fungal biology. The imminent complete genome sequencing of *Neurospora* will allow systematic characterization of clock-controlled genes and detailed studies of the underlying mechanism that generates different phases of gene expression.

Still more genes from the plant world

Clocks sometimes cease to operate in constant light (for example, *Drosophila* and *Neurospora*), but this is not true for plant oscillators^{75,76}. Furthermore, under constant illumination the pace of the plant oscillator is fine-tuned by both the quality (wavelength) and the fluence rate (intensity) of incident light⁷⁷. In addition, as seen in animals, plants use the oscillator to measure photoperiod and show seasonal rhythms, such as the photoperiod-sensitive transition to flowering⁷⁸. Given such a complex interplay between the clock and light, forward genetic screening for plant rhythm mutants under constant darkness would seem to be an obvious approach to the heart of the oscillator. By contrast, simple and robust rhythm assays in constant light, in conjunction with genetic studies of light responses and flowering, has advanced our understanding of the interaction between light and clocks in plants.

Several genes that are implicated in circadian clock function have been identified in *Arabidopsis*, and we are just beginning to understand the underlying molecular interactions (TABLE 4; FIG. 4) Two single MYB-DOMAIN-containing transcription factors — *CCA1* and *LHY*, which share extensive sequence similarity — were independently shown to be associated with the circadian oscillator^{79,80}. Endogenous message and protein levels of both genes cycle with a peak phase of expression at dawn. Either gene, when constitutively overexpressed, will block the overt rhythm under constant conditions of light or dark — as if the clock has stopped running. Furthermore, in overexpressing lines, endogenous message levels of both *CCA1* and *LHY* are severely reduced and show no rhythmicity — offering the first clue that transcription of both *CCA1* and *LHY* might be negatively regulated by their own protein products^{79,80} (FIG. 4).

The mechanism of this feedback regulation became clearer after cloning and characterization of the *TOC1 (TIMING OF CAB EXPRESSION 1)* gene. Mutations at the *TOC1* locus cause period shortening of circadian rhythms that is independent of light (TABLE 4). The *TOC1* protein has features suggestive of an atypical RESPONSE REGULATOR (it is similar to the receiver domains

CHROMOPHORE
A light-absorbing molecule, such as pterin or retinal. Often physically associated with a protein partner to form a photoreceptor/phototransducer.

MYB DOMAIN
A structurally conserved DNA-binding domain found in various transcription factors. In plants, MYB proteins are ubiquitous and known to function in many regulatory systems, including secondary metabolism, cell morphogenesis, the cell cycle, and circadian rhythms.

RESPONSE REGULATOR
Works in conjunction with a sensor kinase that might be activated by an environmental signal. Activation and autophosphorylation of the sensor kinase promotes phosphorylation of a specific response regulator. The latter is often a transcription factor with activity that is modulated by phosphorylation.

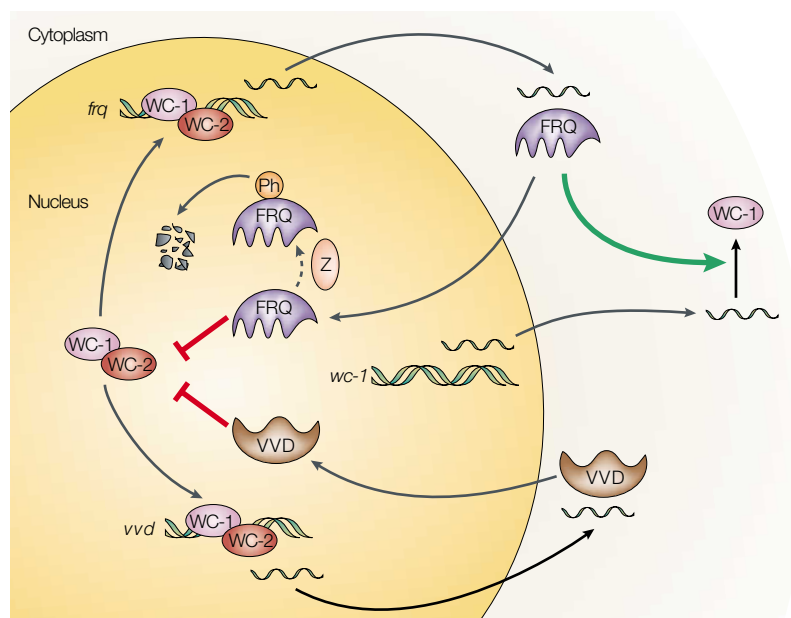


Figure 3 | Elements of the *Neurospora* clock. WC-1 and WC-2 form a protein complex (white-collar complex, WCC) that promotes transcription of *frq* and *vvd*. WCC binds to the *frq* promoter, and possibly activates *vvd* in a similar fashion. The FRQ and VVD proteins independently suppress activity of WCC. The *wc-1* and *wc-2* genes are constitutively transcribed, but accumulation of the WC-1 protein from *wc-1* RNA is stimulated by FRQ through an unknown post-transcriptional mechanism. Unlike the metazoan mechanism, there is no evidence for regulated subcellular localization of any *Neurospora* clock protein. Instead, delays that promote molecular oscillations are believed to depend on controlled degradation of FRQ proteins (dashed arrow). FRQ degradation requires processive phosphorylation by an unidentified kinase(s) (Z). Ph, phosphate.

of response-regulator proteins, however, a key residue essential for phosphorelay is missing). TOC1 also contains a carboxy-terminal motif with high similarity to the **CONSTANS** family of plant transcription factors. Its mRNA level cycles with a peak phase of expression during early evening, which is almost opposite to that of *CCA1/LHY*⁷⁵. This observation immediately prompted a test of whether *TOC1* and *CCA1/LHY* constitute a feedback loop. Interestingly, the *TOC1* message level is constant and low in lines that constitutively overexpress either *CCA1* or *LHY*, which indicates possible transcriptional repression by *CCA1/LHY*⁸¹. The promoter region of *TOC1* contains a *cis*-acting evening element that is also shared by several clock-controlled genes with peak expression in the evening. The element is also similar to a previously described *CCA1*-binding site^{82,83}, which is necessary and sufficient for rhythmic expression of *CCR2* (REF. 82). Recently, Alabadi *et al.*⁸¹ showed a direct and specific binding of *CCA1/LHY* to this evening element, which might repress *TOC1* expression (FIG. 4).

To complete this loop, it is again tempting to test whether *TOC1* regulates *CCA1/LHY* expression. Some evidence for such regulation comes from *toc1* mutants. The reference allele, *toc1-1*, causes a single amino-acid change, and is semi-dominant, whereas the recessive *toc1-2* allele is a splice-site mutation that severely reduces the level of correctly spliced *toc1* mRNA. Interestingly, the expression of both *CCA1* and *LHY* in

toc1-2 homozygotes is reduced, which indicates that functional *TOC1* promotes their transcription, and that reduced levels of both *CCA1* and *LHY* might contribute to the short-period phenotype of *toc1*. This fits well with another observation: a loss-of-function mutation of *cca1*, which still retains *LHY* function, shows a short-period rhythm that is similar to the *toc1* phenotype. *TOC1*, therefore, probably acts as a positive element in the oscillator that promotes transcription of negative elements *CCA1/LHY* (FIG. 4).

Additional positive factors. *CCA1* and *LHY* seem to have some overlapping functions in the negative limb of the oscillator (FIG. 4). Complete loss of either gene or attenuated expression of both of them (as in the *toc1-2* mutant) affects period, but does not abolish rhythmicity^{75,84}. Similarly, persistence of rhythms in the *toc1-2* allele (effectively a null allele, above) indicates the possible presence of some other positive factor that can still sustain expression of *CCA1* and *LHY*, and consequently drive the clock in the absence of *TOC1* function. At the amino-acid sequence level, four additional *Arabidopsis* proteins show significant sequence similarity to *TOC1*. Interestingly, RNAs that encode all four of these proteins accumulate rhythmically. At least one of these genes, cycles with the same phase as *TOC1*, and is therefore a possible candidate for redundant *TOC1* function⁸⁵.

CCA1 function might be regulated by phosphorylation. The protein kinase CK2 phosphorylates *CCA1*, and overexpression of this kinase produces period defects that are consistent with altered *CCA1* activity⁸⁶. Although it is tempting to make comparisons with metazoan and fungal clocks, the biochemical role of clock protein phosphorylation is only beginning to be explored in plants.

The *elf3* mutant raises the possibility of yet another component of the positive limb of the oscillator. *elf3* loss-of-function mutants show arrhythmia only under constant light, while maintaining a functional, albeit low-amplitude, clock under constant darkness⁸⁷. Under constant light, *elf3* plants accumulate a constitutively low level of *CCA1* and *LHY* mRNA and a high level of *TOC1* mRNA, which is consistent with a negative role of *CCA1/LHY* in *TOC1* regulation⁸¹. However, the constant low level of *CCA1/LHY* indicates failure of *TOC1* protein to promote *CCA1/LHY* expression in the absence of functional *ELF3*.

ELF3 RNA and protein levels cycle with a phase similar to that of *TOC1* mRNA, and it codes for a novel protein with no recognizable sequence motifs⁸⁸. Lines that overexpress *ELF3* retain a functional clock, although with a longer period under constant light. This strongly indicates that the mere presence, but not cycling of *ELF3*, is sufficient for sustained rhythmicity⁸⁹. Support for a role of *ELF3* in the oscillator comes from two further lines of investigation that have examined oscillator function in constant darkness. These have established a crucial role for *ELF3* in progression of the clock through early night^{89,90}. Cycling levels of *ELF3* might also have a separate role in attenuating light responses (see below).

Table 4 | ***Arabidopsis* circadian rhythm genes**

Gene name	Properties	References
<i>CIRCADIAN CLOCK ASSOCIATED 1</i> (<i>CCA1</i>)	Loss of function alters, but does not abolish rhythmicity. Overexpression stops clock and suppresses <i>CCA1</i> , <i>LHY</i> and <i>TOC1</i> expression. RNA and protein cycle. Light induces expression. Myb-domain-containing transcription factor with homology to <i>LHY</i> .	79,83
<i>LATE ELONGATED HYPOCOTYL</i> (<i>LHY</i>)	Loss of function alters, but does not abolish rhythmicity. Overexpression stops clock and suppresses <i>CCA1</i> , <i>LHY</i> and <i>TOC1</i> expression. RNA and protein cycle. Light induces expression. Myb-domain-containing transcription factor with homology to <i>CCA1</i> .	80
<i>TIMING OF CAB EXPRESSION 1</i> (<i>TOC1</i>)	Mutations reduce RNA levels of <i>LHY</i> and <i>CCA1</i> . Thought to be a positive regulator of <i>LHY</i> and <i>CCA1</i> . <i>CCA1</i> and <i>LHY</i> bind to <i>TOC1</i> promoter. <i>TOC1</i> RNA cycles. Response regulator homologue.	75
<i>EARLY-FLOWERING 3</i> (<i>ELF3</i>)	Mutations suppress rhythmicity, decrease <i>CCA1</i> and <i>LHY</i> expression and increase <i>TOC1</i> expression in constant light, but not constant dark. <i>ELF3</i> RNA and protein cycle. <i>ELF3</i> attenuates general light responses.	87,88
<i>ZEITLUPE</i> (<i>ZTL</i>)	Mutations affect rhythms in a fluence-rate-dependent fashion. RNA does not cycle.	63

Light responses. Plant oscillators respond to phase-resetting stimuli of light (non-parametric entrainment) in a phase-dependent manner. Light acutely induces transcription of both *LHY* and *CCA1*, although it does not have any immediately detectable effect on the *TOC1* transcript level^{75,79,80}. Acute induction of *CCA1/LHY* might reset its phase of expression, and ultimately the phase of the oscillator. The *CCA1/LHY* response can be superficially compared with clock resetting in *Neurospora*, in which a resetting light pulse induces expression of a negative clock component (*frq*) that usually peaks during subjective day.

Under continuous illumination, light constantly makes phase adjustments (parametric entrainment) and the measured period length is a product of both the intrinsic period of the clock and the cumulative effect of phase resetting by light. Plants show a negative correlation between the period length and ambient light intensity; period lengthens with decreasing light intensity. So, plants that are deficient in photoreceptor(s) show longer period length. Using this model, a role in circadian photoreception was unequivocally ascribed to cryptochromes in plants⁷⁷. Although metazoan cryptochromes can be involved in the core oscillator of at least some tissue types, plant cryptochromes might not have any essential roles in the clock, because *Arabidopsis* deficient in both of its cryptochromes maintains its circadian rhythmicity. Using similar criteria, four PHYTOCHROMES (**PHYA**, **PHYB**, **PHYD** and **PHYE**) have also been implicated as circadian photoreceptors (reviewed in REF. 91).

Several proteins have been identified as potential signalling components downstream of phytochromes and cryptochromes. PIF3, a PAS-domain-containing basic-helix-loop-helix (bHLH) transcription factor, can directly bind to the *CCA1* and *LHY* promoters and mediate their light-induced transcription⁹². **CRY1** and **PHYB** have been shown to enter the nucleus in response to light perception, and then physically associate⁹³. **PHYB** has also been shown to physically interact with **ELF3** (REF. 94).

As in *Neurospora*, plants have mechanisms to restrict the sensitivity of their clocks to light. **ELF3** attenuates general light responses and is produced with a circadian rhythm. So, **ELF3** dynamically regulates the impact of light on the clock, like **VVD** does in *Neurospora*^{87,89} (FIG. 4). Also, **ZTL** (TABLE 4) seems to regulate the well-established effect of light on period length⁶³.

In this comparison of clocks across the kingdoms, it might be significant that plants seem to use a core strategy of gene autoregulation to produce molecular oscillations, but the underlying factors so far have redundant functions. In no instance does loss of a single element of the mechanism eliminate rhythmicity. Plants also use a sophisticated light-entrainment strategy comprised of several photoreceptors that might fine-tune the clock to different ambient light conditions. These features tend to distinguish clocks of the plant world.

A bacterial clock

Although the regulated movement of transcription factors between the nucleus and cytoplasm is probably an essential feature of the metazoan clock, studies of *Neurospora* rhythms have indicated that this cannot apply to all eukaryotes. In fact, work in the late 1980s and early 1990s showed that, in some organisms, a differentiated nucleus is not required at all to organize a circadian clock: circadian rhythms of nitrogenase fixation and the uptake of certain amino acids had been observed in prokaryotic cyanobacteria (reviewed in REF. 95).

A saturation mutagenesis screen in the photosynthetic bacterium *Synechococcus elongatus* led to the identification of more than 100 mutants with aberrant rhythm phenotypes⁹⁶. Many of these mutants have been rescued by wild-type DNA from the *kai* locus (*kaiten* in Japanese means rotation or cycle), which consists of a cluster of three adjacent genes, *kaiA*, *kaiB* and *kaiC* (TABLE 5). Nearly 30 rhythm mutations have been mapped by DNA sequencing to these three genes. The largest gene, *kaiC*, is defined by a series of mutant alleles that generate long-period, short-period, or arrhythmic

PHYTOCHROME
One of three classes of known plant photoreceptors. Composed of a protein moiety covalently associated with a tetrapyrrole chromophore. Synthesized in a red-light-absorbing form, nascent phytochromes are converted by red light to a far-red-absorbing isoform that might have altered stability and function. All phytochromes include carboxy-terminal PAS domains.

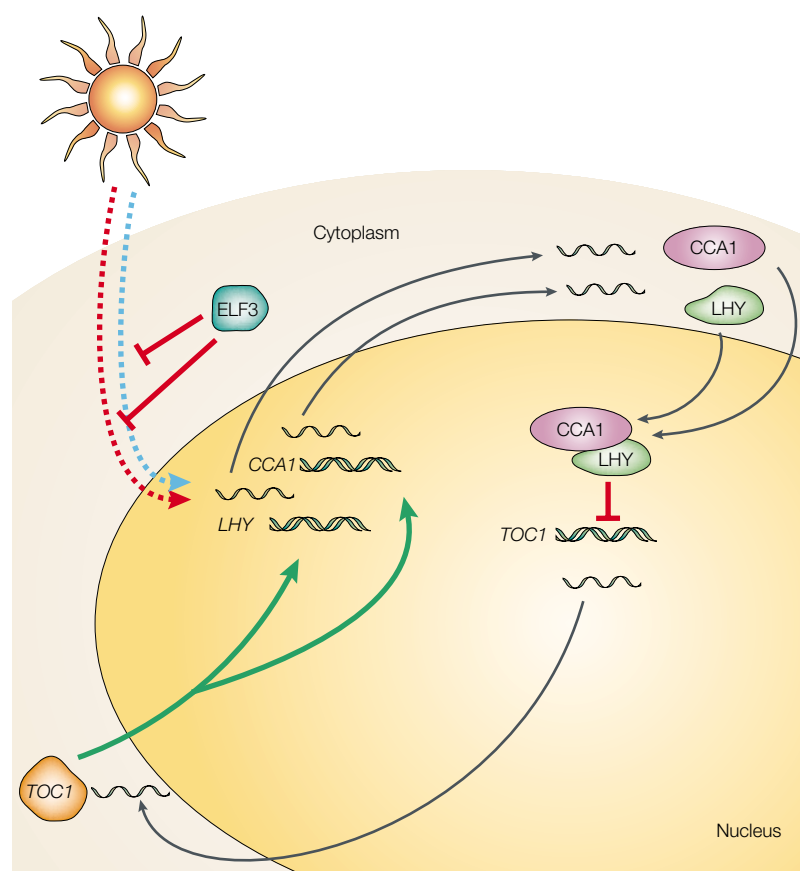


Figure 4 | Regulatory interactions in the *Arabidopsis* clock. CCA1 and LHY are two Myb-motif-containing transcription factors, which directly bind to the *TOC1* promoter and repress its transcription. *TOC1* is an atypical response regulator that acts as the positive element of the clock by promoting transcription of *CCA1* and *LHY* by an unknown mechanism. Both red- and blue-light photoreceptors mediate light-induced phase adjustment by primarily regulating transcription from *CCA1/LHY*. Many photoreceptors are clock controlled at the transcriptional level. Loss of any given photoreceptor or of ELF3 affects clock function only under a specific light regime. ELF3 is a novel clock-controlled protein that acts downstream of all photoreceptors and attenuates light input to the clock. As circadian rhythmicity persists in constant dark in the absence of ELF3, its function is possibly restricted to light interaction with the clock.

phenotypes, and null mutations for *kaiA* and *kaiB* abolish rhythmic phenotypes, which indicates deep involvement in the bacterial clock⁹⁷.

None of the Kai proteins has any known DNA-binding motif, and no genuine homologue of any Kai protein has so far been identified in a eukaryote. Transcription of all three genes is rhythmic, with mRNA levels peaking near the end of the day. Whereas *kaiA* is transcribed as a monocistronic mRNA, *kaiB* and *kaiC* are co-transcribed as a dicistronic message from the *kaiBC* promoter. Ectopic overexpression of *kaiC* suppresses expression from the *kaiBC* promoter, whereas overexpression of *kaiA* enhances expression of *kaiBC*. Loss of *kaiA* leads to a marked decrease in *kaiBC* expression⁹⁷. Therefore, KaiA acts as a positive element and KaiC acts as a negative element in an autoregulatory circuit of some sort (FIG. 5). The KaiB and KaiC proteins show circadian oscillations, both peaking in the early evening (several hours after the peak of mRNA levels), whereas the levels of KaiA show no appreciable circadian variation⁹⁸.

P-LOOP MOTIF
Phosphate-binding domain (P-loop) associated with many ATP- and GTP-binding proteins. These usually have a structure composed of a glycine-rich sequence, followed by certain conserved lysine and serine or threonine residues.

The biochemical roles of the Kai proteins are unclear. However, several assays have shown both homotypic and heterotypic physical associations among the Kai proteins. These interactions are likely to have a role in clock function. For example, physical association of KaiB and KaiA is enhanced by a mutation of KaiA that generates long-period phenotypes^{99,100}. More is known about KaiC. This protein seems to be internally duplicated, each half containing an ATP/GTP-nucleotide-binding Walker A or P-LOOP MOTIF. Single amino-acid substitutions in P-loop-1 can reduce ATP binding by KaiC *in vitro* while generating arrhythmicity *in vivo*¹⁰¹. P-loop-containing proteins often show autokinase activity, and although it is not yet known whether KaiC acts as an autokinase *in vivo*, it has been suggested that KaiC phosphorylation might affect clock function by determining the stability or interaction of the protein with other components of the clock¹⁰¹. KaiC also physically associates with a histidine protein kinase, SasA. This interaction is partly mediated by the amino terminal, 97 amino-acid residues of SasA. Interestingly, these 97 residues share high sequence similarity with the 102-residue KaiB protein¹⁰². Loss-of-function mutants of *sasA* show no rhythmicity at high light intensity, whereas low-amplitude rhythms persist under low-intensity light, implying a role for SasA in the clock and in perception of light intensity¹⁰². Alternatively, the light sensitivity of the rhythms might result indirectly from a broader defect in the circadian regulation of downstream genes, or from destabilization of Kai protein complex(es) owing to the absence of SasA (see below). A more specific role for SasA in the clock itself has also been proposed: from its structure, SasA is predicted to work in conjunction with an unidentified response regulator¹⁰². Phosphorylation and dephosphorylation of such a response regulator might be influenced by varying the physical associations of KaiB, KaiC and SasA. This could serve as a reversible switch to affect expression of *kaiBC* (FIG. 5). Indeed, immunoprecipitation of KaiC protein at two points during a circadian cycle showed SasA as a constitutive KaiC partner and KaiB as an oscillatory partner¹⁰². These data are consistent with a clock model whereby the efficiency of the SasA–KaiC interaction is mediated through time by the KaiB–KaiC protein interaction.

An exhaustive survey of circadian gene expression in cyanobacteria yielded surprising findings. When a promoterless luciferase reporter gene was inserted randomly into the cyanobacterial genome, all of the resulting 800 bioluminescent strains showed a circadian rhythm in light production¹⁰³. Such broad rhythmicity of gene expression indicates that the cyanobacterial clock might regulate the factors that compose the general transcriptional machinery of these organisms or might influence the structural features of the chromosome. Is such a profound coordination of gene activity really of value to the organism? This is beginning to be addressed in population studies that mismatch genetically determined rhythms and environmental cycles of light and dark. In co-culture, those bacteria whose endogenous circadian rhythms best match the external light–dark

Table 5 | **Cyanobacteria circadian rhythm genes**

Gene name	Properties	References
<i>kaiA</i>	Mutations alter rhythmicity. RNA, but not protein, cycles. Accumulation of KaiA promotes expression of <i>kaiBC</i> . Physically associates with KaiB and KaiC.	96,97
<i>kaiB</i>	Mutations alter rhythmicity. RNA and protein cycle. Transcribed with <i>kaiC</i> as part of a dicistronic message. Physically associates with KaiA and KaiC. Mutations of <i>kaiA</i> enhance KaiA/KaiB interaction.	96,97
<i>kaiC</i>	Mutations alter rhythmicity (arrhythmic, short- and long-period alleles). RNA and protein cycle. Transcribed with <i>kaiB</i> as part of a dicistronic message. Transient overexpression phase-shifts clock. Nucleotide-binding protein with autokinase activity. If overproduced, KaiC suppresses expression of <i>kaiBC</i> . Physically associates with KaiA and KaiB.	96,97
<i>Synechococcus adaptive sensor (sasA)</i>	Mutations alter rhythmicity, but null does not block rhythmicity. Transient overexpression phase-shifts clock. Histidine kinase with KaiB-like sensory domain. Physically associates with KaiC.	102
<i>Circadian input kinase (cikA)</i>	Null mutations shorten period, affect the phases of some rhythms, and alter entrainment to dark pulses. Bacteriophytochrome and histidine protein kinase homologies. Possibly involved in photo-entrainment of the clock.	141

cycle always supplant cultures with mismatched rhythmicity. In the absence of environmental cycles, these growth differences are not observed¹⁰⁴. Evidently, the specific period of the natural timing mechanism strongly enhances survival in light–dark cycles that correspond to a solar day. *Synechococcus* might soon provide answers to even larger questions that concern the fitness of truly clock-less organisms and those specific elements of physiology that are most dependent on endogenous rhythmicity.

Primordial timepiece?

Are all present-day clocks derived from a common ancestral mechanism? Given the unique character of all clock proteins found in cyanobacteria, prokaryotic and eukaryotic clocks are very likely to have arisen independently. Additionally, there is no evidence that any Kai protein acts at the level of transcription to influence cycling *kai* gene expression. A new form of autoregulation might underlie the cyanobacterial clock.

There is also much in favour of independent origins for plant, fungal and metazoan clocks. The metazoan clock genes have not turned up in screens for circadian rhythm mutants in other kingdoms. Perhaps more significantly, complete sequencing of the *Drosophila* and human genomes has failed to identify clear orthologues of any plant or fungal clock protein. And we have described fundamental differences in the way some well-studied eukaryotic clocks promote rhythmicity. Clock proteins are subject to a complex cytoplasmic regulation in the animal kingdom that is not observed in *Neurospora*. Casein kinase 1 ϵ affects the stability of newly translated cytoplasmic PER proteins in flies and mammals, and cytoplasmic heterodimerization with a partner clock protein determines subcellular localization of PER and its sensitivity to CK1 ϵ . In *Drosophila*, certain rhythm mutations specifically affect patterns of cytoplasmic heterodimerization of PER and TIM to change period length^{22,105}. A different group of mutations blocks or changes the rate of nuclear translocation of PER/TIM

complexes^{12,106}. A third class specifically affects the rate of disassembly of PER/TIM complexes after nuclear transfer¹⁷. The key regulation in fungi seems focused on the timed disappearance of nuclear proteins.

The connection between plants and fungi does not seem to be any stronger. There are no significant homologies between the known plant and fungal clock genes and, as we have discussed, plant clock genes seem to carry out only redundant functions in a molecular mechanism that is still poorly understood. Sequencing of the *Arabidopsis* genome has not shown convincing orthologues of *frq*, *wc-1* and *wc-2*.

The presence of PAS domains, and occasional neighbouring sequence similarities, in several plant, fungal, and metazoan clock proteins, is probably the strongest argument for a common ancestral clock. Although there are various ways in which clock proteins might interact, the animal and fungal oscillators rely on transcriptional activators that contain PAS motifs to organize their autoregulatory networks. However, there are difficulties in weighing such evidence. One lesson comes from a related search for protein homologies across the plant and animal kingdoms. Plants and animals are generally agreed to have sprung from a common, single-celled ancestor. However, in both groups of organisms, we find homeobox-containing proteins that make fundamental contributions to development. As multicellular organization and molecular systems of development must have evolved independently in plants and animals, it would be a mistake to conclude that sequence similarities among plant and animal homeoboxes reflect derivation from a common developmental programme.

Sampling from a limited set of primitive transcription factors might explain the present-day distributions of both homeodomain and PAS-containing proteins. But it seems more likely that certain attributes of both classes of protein stacked the deck a long time ago. PAS motifs in the aryl hydrocarbon receptor are involved in toxin binding, indicating a more generalized potential for binding small ligands⁶¹. It has already been noted

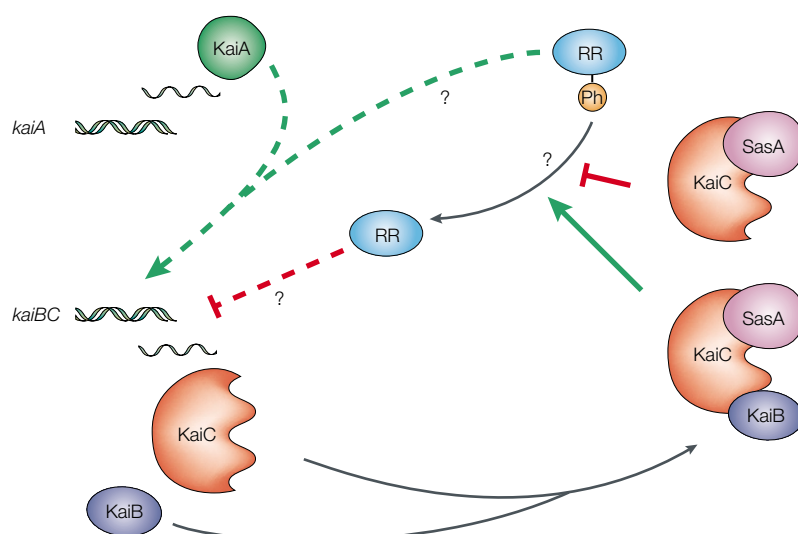


Figure 5 | Possible regulation in the *Synechococcus* clock. High levels of KaiA somehow promote, and high KaiC inhibits (not depicted), transcription of a dicistronic mRNA from *kaiBC*. Physical interactions have been observed among all three Kai proteins, and between KaiC and SasA. The specific role of SasA is unknown, but it might function in conjunction with an unidentified response regulator (RR). It has been suggested¹⁰² that high levels of a KaiB/KaiC complex might promote dephosphorylation by SasA of such an RR to suppress *kaiBC* expression, and that in the absence of KaiB/KaiC complexes, SasA/KaiC complexes might promote phosphorylation of this RR (giving RR-Ph) to enhance *kaiBC* expression. Broken lines indicate that the proposed regulatory events could require many unidentified, intermediate factors. Ph, phosphate.

that some PAS-containing proteins have properties that might accommodate construction of light-sensitive circadian clocks. WC-1 and WC-2, the phytochromes and phototropins of plants, and bacterial PHOTOACTIVE YELLOW PROTEIN are photo-receptors, and the latter two types of protein might depend on PAS-related sequences to carry an associated chromophore^{64,91}. Recently, it was shown that retinoid receptors can physically associate with human CLOCK (or its paralogue MOP4), to inhibit CLOCK/BMAL1- and MOP4/BMAL1-dependent transcription in the vasculature¹⁰⁷. It has also been discovered that heterodimerization of BMAL1 with CLOCK (or the CLOCK paralogue NPAS2, which functions in forebrain) is sensitive to the relative concentrations of reduced and oxidized NAD in the cell¹⁰⁸. Recall that in hepatocytes, *Per* RNA cycles are efficiently reset by altering feeding schedules in rodents^{56,57}. This resetting was shown to be independent of the effects of light on the SCN. So, in present-day clocks, redox sensitivities might establish mechanisms for metabolic entrainment¹⁰⁸.

This latter work especially indicates several new questions that might bear on the evolutionary origins of molecular clocks. In ancestral cells, a large family of PAS-containing proteins could have emerged to broadly coordinate transcription, environmental/developmental responsiveness and metabolism, while enabling the later, independent assembly of sensorial molecular oscillators. Are redox or hormone responses found for paralogues of CLOCK and BMAL1 that do not have circadian function? For example, it has

already been shown that interactions of CLOCK and NPAS2 with the bHLH PAS protein ARNT retain redox sensitivity¹⁰⁸. ARNT has no known role in the clock, but is a well-established partner of the aryl hydrocarbon receptor⁶¹. It has been suggested that BMAL1 and *Neurospora*'s WC-1 evolved from a common ancestral clock protein⁶². Although redox control depends on bHLH sequences that adjoin PAS in CLOCK, NPAS2 and BMAL1, some of these sequences might have been conserved in WC-1 (REF. 62). Is there a vestige of redox sensitivity in WC-1? Complex interactions of metabolism and the clock are already well documented in *Neurospora*¹⁰⁹, and it has been argued that FRQ, WC-1 and WC-2 might provide a mechanism for entraining a larger metabolic oscillator¹¹⁰. Will associations between WC-1 and WC-2 be found to be redox sensitive in fungi?

Revisiting the human perspective

Studies of FASPS not only showed the value of comparative genetics, they brought a new set of questions to the table. So far, this syndrome has been defined in terms of its effects on patterns of sleep and other aspects of circadian function. However, pinpointing a single affected gene brings further questions about phenotype. Are there broader physiological and behavioural responses to an altered clock or defective *PER2* action? For example, mutations of *per* in *Drosophila* have been linked to robust changes in certain drug sensitivities and to defects in the patterns of expression of some genes that are tightly connected to the control of learning and memory^{111,112}.

We are also now in a position to take a much more focused approach to candidate gene mapping. Work in several systems has produced the list of genes, the variations of which are to be tabulated. Although the specific sequence change associated with *PER2* in FASPS tells us how an extreme, but rare, phenotype can be generated, in the long run more subtle differences might prove to be far more interesting. Self-proclaimed night owls and morning larks are easy to find. Will their behaviour always prove to be a simple matter of self-discipline, or might variations in established genes link a small group of common polymorphisms to these behavioural preferences? We are already aware of naturally occurring diversity in behaviour and *per* gene structure in *Drosophila*¹¹³. Perhaps subtle, genetically determined variations in human wake–sleep behaviour will turn out to be no less common than differences in hair or eye colour. Such a result should not be surprising if the overall shape of human wake–sleep behaviour is so deeply carved by our genes.

Links

DATABASE LINKS Familial advanced sleep phase syndrome | *PER2* | *frequency* | Timeless | Clock | Cycle | Double-time | *cryptochrome* | *shaggy* | *vriille* | Armadillo | Dishevelled | CLOCK | ARNTL | *Clock* | *Per* | *Arntl* | *Cry1* | *Cry2* | CCA1 | *TOC1* | CONSTANS | *elf3* | PHYA | PHYB | PHYD | PHYE | CRY1 | ARNT

PHOTOACTIVE YELLOW PROTEIN

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