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# CIRCADIAN OSCILLATORS IN EUKARYOTES

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## **Abstract**

The biological clock, present in nearly all eukaryotes, has evolved such that organisms can adapt to our planet's rotation in order to anticipate the coming day or night as well as to anticipate unfavorable seasons. As all modern high-precision chronometers, the biological clock uses oscillation as a timekeeping element. In this review we describe briefly the discovery, historical development, and general properties of circadian oscillators. The issue of temperature compensation is discussed and our present understanding of the underlying genetic and biochemical mechanisms in circadian oscillators are described with special emphasis on *Neurospora crassa*, mammals and plants.

## **Keywords**

*Circadian oscillators, biological clock, temperature compensation, clock genes, transcription, translation, feedback regulation, photoperiodicity*

## **Introduction**

Androstheneas from Thasus, a member of an expedition sent out by Alexander the Great, made first systematic observations on diurnal rhythms in plants. Although his original report is lost, fragments describe that during his journey he observed astonishing leaf rhythms in *Tamarindus indica* which suggested to him that these trees were sleeping during the night [1, 2]. The first modern report that leaf rhythms are endogenously generated date back to de Mairan, an astronomer, who showed that leaf rhythms in *Mimosa* plants continued even in the absence of an external light/dark cycle [3]. De Mairan's studies were quickly followed-up, as for example by the physician Zinn on 'plant sleep' [4], or by Linnaeus' famous 'flower clock' described in his *Philosophia Botanica* [5]. In the beginning of the 19<sup>th</sup> century the pharmacist Julien-Joseph Virey found that human mortality shows daily and seasonal variations. Virey also reported on the effect of drugs with respect to their administration times, and appears therefore to be the first person working in the field which now is called "chronopharmacology" [6].

However, the endogenous character of plant leaf movements was not universally accepted. Wilhelm Pfeffer, while trying to demonstrate that leaf movements in bean plants were caused by environmental influences, showed by well-designed experiments, that these

oscillations indeed have an endogenous cause [7]. During the same period, similar findings were made by Szymanski [8] on animals.

In the 1930's Erwin Bünning suggested that intracellular time measurement leads to seasonal adaptations, such as flower induction, migration and hibernation, which is based on an oscillatory and genetically determined physiological clock with a period of approximately one day. While Bünning's hypothesis first caused major opposition, it became generally accepted during the 1950's [9, 10]. His textbook "The Physiological Clock" [10] still makes an interesting introduction to the field.

Today, the name *circadian* indicates that under free-running conditions the period length of these physiological oscillators is *circa* one day (derived from lat. *dies*, day and *circa* about) after a suggestion by Franz Halberg. Additional defining properties of circadian oscillators are: (i) being endogenously generated; (ii) showing a free-running rhythm; (iii) can be phase-shifted by environmental perturbations, for example by light, temperature, chemicals; (iv) they show entrainment, i.e., circadian oscillators can track rhythmic environmental changes, and (v) show temperature compensation, meaning that the free-running period is (approximately) the same at different but constant temperatures.

Circadian rhythms are important for the daily and seasonal adaptation of practically all higher (eukaryotic) organisms, but are also found in light-sensing prokaryotes such as cyanobacteria [11]. However, adaptation of organisms to their environments does not only involve circadian oscillations, but also ultradian as well as infradian oscillators [10, 12-16].

In this review we give a brief description of eukaryotic circadian oscillators with special emphasis on the model organisms *Neurospora crassa*, *Arabidopsis thaliana* and the mammalian clock. *Drosophila*, while a major model system, is left out here due to space limitations.

## **Genetics and Model Organisms**

In the beginning of the 1970's [17] the first successfully generated clock mutants were generated with the fruit fly *Drosophila melanogaster* [18] and the filamentous fungus *Neurospora crassa* [19], and rats were found to lose their circadian rhythms by hypothalamic or suprachiasmatic lesions [20, 21]. Remarkably, in 1990 Ralph et al. could restore circadian wheel-running activities in Syrian hamsters that had their suprachiasmatic nucleus (SCN) removed, by transplanting back intact SCN tissue [22], indicating that the mammalian circadian clock is located in the SCN [23].

Early genetic and molecular biology studies on *Drosophila* [24] and *Neurospora* [25] indicated a common mechanism involving a transcriptional translational negative feedback loop (Fig. 1) [26-30], but newer findings suggest the presence of multiple loops and oscillators [31-36].

## **Circadian oscillators are based on feedback mechanisms**

The study of biological clocks had always a good share of theoretical studies and modeling approaches [37-39]. Kinetic models of transcriptional-translational negative feedback loops, some based on Goodwin's equations [40, 41], showed that many aspects of circadian oscillations including temperature compensation and phase resetting can be described [38, 42-59]. Early predictions using the Goodwin oscillator indicated [42, 60] that clock protein stability/turnover should determine the circadian period length, where short period mutants should have a clock protein which is more rapidly turned over compared with wild-type, while in long period mutants the clock protein should be more stable than in wild-type. Using *Neurospora*,

it was demonstrated that phosphorylation of the clock protein FREQUENCY (FRQ) is important for its stability [61-64]. When certain phosphorylation sites in FRQ were blocked (for example replacing Ser 314 by an Ile) [62], FRQ stability increases and leads, as theoretically predicted [42, 60], to larger period lengths. In several follow-up papers by the Liu group [65-67], it was found that phosphorylated FRQ is turned over by the ubiquitin-proteasome pathway [68]. The study of FRQ-decay kinetics in *Neurospora* clock mutants confirmed the theoretically predicted period-stability relationship with an intimate link to temperature compensation [53, 64]. Thus, *Neurospora's* circadian period appears to be a fine-tuned process including phosphorylation / dephosphorylation reactions of FRQ by several kinases and phosphatases, leading to a regulated turnover through the ubiquitin-proteasome pathway [27, 28, 69-72]. Similar observations have also been made for mammalian systems showing that the decreased period for the CK1 $\epsilon$  *tau* mutation in mice and Syrian hamsters is related to an increased degradation in PER-protein [73, 74]. Certain posttranslational regulation elements of clock proteins appear to be conserved from *Neurospora* to mammals and involve the kinases CK1 and CK2 and the phosphatase PP2A [69].

Positive feedback loops (Fig. 1) have also been identified as part of circadian clock mechanism, as for example in *Drosophila* [35, 75-78]. Some models showed that the presence of interlocked positive and negative feedback loops may increase the stability and tuneability of the oscillator [79], while in other cases [80, 81] the presence of an additional positive feedback did not seem to affect the robustness of the oscillator. In case of the *Drosophila* oscillator, which at present includes two negative and one positive feedback loop, the positive loop is necessary to describe the influence of dosage of the *per*-gene and *vri*- on the period [75, 82-84].

There is a close similarity from a mechanistic/kinetic viewpoint between circadian rhythms and *in vitro* physicochemical oscillators [85-96], as both have positive and negative feedback loops [97]. Today, the mechanisms of many physicochemical oscillators have been determined, including systems that even can show temperature compensation [93, 98, 99].

### **The Issue of Temperature Compensation**

Temperature compensation (TC) is one of the defining clock properties of circadian rhythms. TC means that the circadian period is homeostatically regulated towards variations in temperature, i.e., the circadian period is constant at different (constant) temperatures. TC is only operative within a certain, for the organism important temperature range. For most of the circadian oscillators the precise mechanism how TC is achieved is still not known. A variety of suggestions how TC may be achieved have been considered during the years [38, 51].

In the 'balancing/opposing reaction approach', first suggested in 1957 [100], then later kinetically formulated for chemical oscillators [54], each temperature-induced change in a rate constant of a reaction step will in principle lead to an increase or decrease in the period length. For certain combinations of activation energies the positive and negative influences of the various rate constants on the period length cancel and the system will show TC within a given temperature range. To achieve TC the activation energies need to be fine-tuned in such a way that the sum of the product between the sensitivities and the activation energies becomes zero [51, 101, 102]. This approach allows one to describe TC of any systemic property which depends on the rate constants, such as for non-oscillatory steady-state fluxes or steady-state concentrations [50], and has been extended to describe pH-compensation [103, 104]. Several experimental findings suggest (see below) that 'balancing' is at least one mechanism to achieve temperature compensation in circadian rhythms.

Hong et al. [105] recently argued that a balancing approach would not be sufficiently

robust to account for the many mutations, which do not affect temperature compensation. They propose a switch-like mechanism for circadian rhythms that concentrates period sensitivity in just two parameters, by forcing the system to alternate between a stable steady state and a stable limit cycle. Indeed, there appears to be a close relationship between robust homeostasis and temperature compensation [106], but such a relationship for circadian oscillators is still poorly understood.

Despite TC, temperature has a significant influence on other circadian properties such as entrainment, phase shifting, or amplitude [55].

## **The *Neurospora* Circadian Clock**

### *The FRQ-oscillator*

*Neurospora crassa* is a model organism [107] that has been extensively used in the study of circadian rhythms [27, 28, 69, 70, 108-111]. In 1959 Pittendrigh [112] found that *Neurospora* shows a circadian rhythm in its asexual production of spores (conidia). The use of the *band* (*bd*) mutation introduced later by Sargent and coworkers [28, 107] allowed monitoring of the free-running temperature compensated conidiation rhythm in growth tubes (Fig. 2). A firefly luciferase-based reporter assay was first constructed by Morgan *et al.* [113], where the sequence of the luciferase gene was partly optimized to reflect the codon usage by *N. crassa*. Both light-induced and circadian activities could be continuously monitored using this assay. A fully codon-optimized system was recently generated by Gooch *et al.* [114], which showed a dramatic increase in the light output of the luciferase-catalyzed reaction and which has also been applied to study the output dynamics under conditions of choline deficiency [115] (see also *FRQ-independent oscillators* below).

The basic mechanism behind the conidiation rhythm is due to a transcriptional translational negative feedback loop, where the FRQ-protein inhibits its own transcription (FRQ oscillator, Fig. 3). WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2) are Zn-finger proteins acting as a heterodimeric transcription factor, the so-called White Collar Complex (WCC). The WCC plays central roles in a variety of different physiological processes, including (blue) light activation of genes [107, 116-124], with WC-1 as a flavin-binding blue light photoreceptor. The *frq* promoter contains two light responsive elements (LREs), where the distal element ("clock (C)-box") [125] appears necessary for rhythmicity in darkness. Each LRE contains two GATN sequence repeats, each probably capable of binding the Zn-finger domain from either WC-1 or WC-2. In darkness, circadian rhythms are observed in *frq*-mRNA, FRQ-protein, as well as in WC-1 [126]. Hong *et al.* [127] showed by model calculations that the binding of WCC to the *frq*-promoter is of importance for maintaining temperature compensation. Alternative to a rapid degradation of the complex between FRQ and WCC, in order to close the negative feedback loop, there is evidence for a FRQ-mediated clearance of WC-1 out of the nucleus [128]. Recent experimental evidence suggests that FRQ is rapidly shuttled between the nucleus and the cytoplasm [129], which may be part of a FRQ-mediated mechanism to clear WC-1 out of the nucleus.

While WC-1 has been considered to be always bound to WC-2, which has been found to be in excess compared to WC-1 and at constant concentrations [120, 130, 131], recent ChIP experiments indicate differential binding affinities of WC-1, WC-2 towards the LREs and a breakup of the WCC [132]. It was found that WC-1 is always bound to both LREs, while binding of WC-2 in darkness to the C-box is oscillatory (circadian) and highly correlated with the binding of the chromatin-remodeling enzyme CLOCKSWITCH (CSW-1) to the C-box [132].

Due to a temperature-regulated alternative splicing mechanism, the FRQ-protein is found in a long (l-FRQ) and a short form (s-FRQ). When individually expressed, each form shows temperature compensated oscillations, but together they extend the temperature range for which temperature compensation is observed [133-135]. A recent kinetic model by Akman *et al.* [136] describes the temperature-induced two FRQ isoforms and the associated temperature compensation not only for the *bd* mutant, but also for *frq*<sup>1</sup>, *frq*<sup>7</sup> and *frq*<sup>SS131</sup> mutants.

As already mentioned above, the expressed FRQ-protein (i.e., both the s- and l-form) is post-translationally modified by a variety of kinases as well as phosphatases leading to a fine-tuned stability of the protein, which regulates the period of *Neurospora's* circadian rhythm [63]. Casein Kinase 2 (CK2) has been found to be a key regulator of temperature compensation in *Neurospora* [94]. The *chrono* and *period-3* mutations have been found to be within the  $\beta$ 1 and  $\alpha$  subunits of CK2. Reducing the dose of these subunits, significantly alters temperature compensation indicating that temperature compensation is due to a balancing of positive and negative contributions to the period [137].

Besides regulating FRQ-protein stability by proteasomal degradation [67], there is now evidence that *frq*-mRNA is regulated by the Exosome and defining an additional posttranscriptional negative feedback loop [138].

FRQ dimerizes by a coiled-coil domain, which is important for maintaining circadian rhythmicity [139]. FRQ also binds to a "FRQ-interacting RNA helicase", FRH [140]. Downregulation of FRH using RNA interference has been found to lead to increased *frq*-mRNA levels indicating that FRH is important in the negative loop of *Neurospora's* clock mechanism [72].

When transferring cultures from darkness to continuous light conditions, the circadian rhythm is abolished, *frq*-mRNA and FRQ-protein levels reach a steady state (after partial adaptation responses) and growth tubes show constant conidiation [120, 141, 142]. The light resetting behavior of the *Neurospora* clock which has been characterized by several groups is well described by a Goodwin oscillator using the assumption that light overrides the inhibitory effect of FRQ on its own transcription and increases *frq* transcription [143]. VIVID (VVD) is another light-upregulated and light-responsive protein, which contains a blue light receptor [144-146]. The role of VVD is associated with the control of the phase of *Neurospora's* circadian rhythm, its light resetting and transient light response [144, 147-149] as well as in the temperature compensation of the circadian phase [150]. In the *vvd*<sup>KO</sup> the phosphorylation pattern of FRQ is altered. At DD4 more of the lower-phosphorylated forms are seen in *vvd*<sup>KO</sup> while in the wild-type strain FRQ is hyperphosphorylated [150] indicating that VVD somehow interacts with FRQ and/or FRQ-phosphorylating or dephosphorylating processes. Schneider *et al.* [151] have recently found that a *vvd* mutant strain can show rhythmic conidiation under constant light (LL) condition. The period of this strain ranges between 6 to 21 hr in LL dependent upon the light intensity, the carbon source in the medium and the presence of other mutations. The rhythms in LL require the *wc-1* genes, but not the *frq* gene and FRQ does not show oscillations. Schneider *et al.* [151] therefore conclude that the conidiation rhythm observed in LL in the *vvd* strain is driven by an oscillator independent of FRQ.

### *FRQ-independent oscillators*

Surprisingly, certain circadian or noncircadian oscillations do not seem to require a functional FRQ protein. They are often referred to as "FRQ-less oscillators" (FLOs) [152, 153]. The first strain containing a FLO, *frq<sup>9</sup>*, was characterized by Loros *et al.* [154]. In this strain a complete loss in temperature compensation in its conidiation rhythm was observed. This strain produces a short nonfunctional form of FRQ, and the observed phenotype, showing noncircadian banding appearing after a certain induction time, was confirmed using a true *frq*-knockout strain (*frq<sup>10</sup>*) [155].

Several FLOs have now been identified, and alternative hypotheses for the 'circadian pacemaker' in *Neurospora* have been put forward [151, 152, 156-160]. Many of these FLOs lack one or more of the defining properties of circadian rhythms and are therefore noncircadian [31]. There is presently a disagreement whether some of the FLOs can be entrained by temperature cycles [161-163].

de Paula *et al.* [31, 32] recently found a FLO, which shows circadian (i.e., temperature compensated) oscillations in the activity of the *clock-controlled gene 16 (ccg-16)* both in darkness as well as under continuous light conditions. The oscillator requires WC-1 and WC-2 and there is the possibility that this WC-FLO is involved in the generation of WC-1 rhythms.

When nitrate ion is the only nitrogen source, the nitrate assimilation pathway is turned on showing oscillations in nitrate reductase (NR) activity with a period length of approximately 24 hours [33]. These oscillations do not require a functional FRQ, but do require WC-1, and are observed both in darkness as well as under continuous light conditions. The 'nitrate FLO' contains a negative feedback loop, where the downstream product of NR, the NITROGEN METABOLITE REGULATOR (NMR) protein inhibits the transcription of *nit-3* (the structural gene of NR) by binding to its transcription factor NIT-2 [164, 165]. The existence of such a nitrogen oscillator allows efficient nitrogen uptake at the phase when physiological activity is high.

## **The Mammalian Circadian Clock**

### *The master clock*

Today, the SCN is recognized not only to act as a central clock, but also as a synchronizer of circadian rhythmicity in other tissues [14]. It is now generally accepted that the retina measures the light intensity through a non-image photoreception and transmits this signal to the SCN. This is mediated by the pigment melanopsin [166], which is accepted as a major component in the synchronisation of circadian clocks.

The SCN has efferents to peripheral tissues, which constitutes a part of the sympathetic outflow from the brain to the kidneys, bladder, spleen, adrenal and thyroid glands, as well as to white and brown adipose tissue. The SCN is also involved in the parasympathetic nervous system with innervation of the liver, pancreas, thyroid and submandibular glands. Possibly, there is also a modulation of the neuroendocrine systems, as well [167]. In addition, secretion of melatonin from the pineal gland is regulated through nerve pulses from SCN, whereby the modulatory role of melatonin on the sleep/wake rhythms, blood pressure and other functions is effected via the blood stream [12, 14].

It has also been found that *transforming growth factor alpha* (TGF- $\alpha$ ) functions as an output signal from the mammalian clock in the SCN, mediated through the EGF receptors on the neurons in the hypothalamic subparaventricular zone in mice [168, 169].

### *The cellular clockwork*

There is now increasing evidence that clock genes are expressed in the oocyte and during early embryonic development [170]. The mammalian circadian clock is a complex autoregulatory transcriptional and translational feedback program, which is composed of positive and negative regulators [171]. Two basic helix-loop-helix transcription factors, CLOCK AND BMAL1, form a heterodimer, which constitute the positive elements and drive transcription of three *Period (Per)* and two *Cryptochrome (Cry)* genes (Fig. 4). In the nucleus, the heterodimers bind to E-box enhancer elements in the promoter regions of the genes encoding *Per1*, *Per2*, *Per3*, *Cry1* and *Cry2* and enhance transcription [172]. In intact animals transcription of *Per1* starts before dawn and has a peak in *Per1*-mRNA about 6 hours later. The levels then rapidly subside before the end of the day. The resulting peak of the PER1 protein comes 6 hours after its mRNA. *Per3* transcripts accumulate at the beginning of the day and subside after 4-6 hours, while *Per2* mRNA accumulation occurs later than the two others and peaks at dusk. The transcripts of *Cry1* and *Cry2* reach a peak at 6 to 8 hours after dawn and thereafter decline. In contrast to the transcripts, the resulting proteins all oscillate with the same phasing and reach maximum levels at dusk. The PER and CRY proteins are bound and phosphorylated by a casein kinase 1 epsilon/delta (CK1 $\epsilon/\delta$ ). It has been found that phosphorylation by CK1 $\epsilon/\delta$  is temperature-insensitive and period-determining [173], probably by an "instantaneous" [51] temperature compensation mechanism of the enzyme. In addition, PER and CRY proteins translocate to the nucleus and act as negative regulators, both of their own transcription and by directly interacting with the CLOCK-BMAL1 heterodimer. Their transcription is therefore inhibited during the night (see [171]). It has recently been found that CLOCK possesses intrinsic histone acetyltransferase activity in mouse liver cells, which contributes to chromatin-remodelling events related to circadian control of gene expression. In addition, CLOCK mediates acetylation of BMAL1, which serves as another regulatory element in the clock. Thereby, BMAL1 undergoes rhythmic acetylation in the liver, where the timing parallels the down-regulation of circadian transcription in clock-controlled genes [174].

At least two other proteins may modulate PER1 activity in mammalian cells by regulating the circadian periodicity [175]. In addition, *Rev-erb- $\alpha$*  modulates the clock by prolonging the periodicity and also coordinating metabolic pathways [176]. Light then resets the master clock in the SCN, where the pigment melanopsin plays a central role [166]. However, the effect is depending on the time when it acts, causing both phase shift and modulation of the circadian phase [166, 177]. In addition, at least two different types of microRNA exist which are interacting with the CLOCK-BMAL1 complex, whereby the circadian period is lengthened and the entrainment of the master clock by light is attenuated [176].

### *Peripheral clocks*

The cloning and characterization of mammalian clock genes has revealed that they are generally expressed in a circadian manner in almost all organs of the body [178]. For nearly 30 years it has been known that the rate of cell proliferation undergoes substantial circadian variations, where the phasing differs from tissue to tissue. It has been shown that the molecular circadian clock exerts a direct control on the cell division cycle in proliferating tissues by modulating the activity of cyclins and cyclin dependent kinases [179]. Still, it is not clear what



causes the phase delay in some tissues. On the other hand, rhythms of body temperature in rodents can sustain peripheral circadian clocks, being an indirect mechanism for phase synchronisation [180]. Peripheral clocks appear also important for the regulation of cardiovascular and metabolic functions [181].

Since the 1980s numerous reports have described cyclic variations in different parts of hemopoiesis, both in the maturing compartments of the bone marrow and in the relative numbers of different types of leukocytes in peripheral blood [182-187]. It has been postulated that the whole immune system is both exogenously regulated and controlled by the endogenous clock from SCN [188]. In particular, BMAL1 seems to be important for the development of B cells along a circadian time scale [189]. In line with this, it has recently been reported that the circadian expression of monocyte chemoattractant protein-1 (MCP-1/JE), which is important for the phagocytic functions in macrophages, is directly controlled by BMAL1 [190].

### *Stem cells*

Several years ago, it was shown that the clonability of murine progenitor cells underwent circadian variations when cultured in semisolid medium [191-194]. These variations were synchronous with the proliferative activity of the bone marrow, indicating a general systemic regulation of hemopoiesis. Later, it was shown that the different clock genes were not only expressed in hemopoietic stem cells in mice [195], but also appeared to be developmentally regulated [196]. Subsequent sampling of human stem and progenitor cells (CD34+) from the bone marrow showed a different pattern, both with regard to phasing and amplitude [197]. Maximum mRNA level for *Per1*, *Per2* and *Cry2* were found during the morning, whereas *Rev-erb α*, *Bmal1* and *Clock* did not show significant circadian variations.

Recently, it has been found that hemopoietic cell trafficking is due to regulated adhesion and attraction to the bone marrow microenvironment [198]. In line with this it was reported that hemopoietic stem cell release in mice is regulated through circadian oscillations, peaking at 5 hours after the initiation of light, and reaching a nadir at 5 hours after darkness [199].

Cultured human mesenchymal stem cells from the bone marrow can show circadian rhythms using serum shock [200-202] and cAMP analogs. The phosphorylation status of both PER1 and GSK3β was essential for getting circadian rhythms [203]. Since such stem cells are essential for normal hemopoiesis to take place *in vivo*, this appears to be a promising model for studying molecular networks related to the circadian clocks.

### *Cell culture studies*

During the last decade circadian oscillations have also been observed in mammalian cells from peripheral tissues, and mainly in murine and rat fibroblasts (for review, see [204]). It was shown that serum shock induced the circadian expression of various clock genes both in fibroblasts and hepatoma cells from rats [202]. Later it was shown that cAMP, protein kinase C, glucocorticoid hormones and Ca<sup>2+</sup> had the same effect [200]. Surprisingly, it was found that multiple signaling pathways in the cells could elicit circadian gene expression [201].

Importantly, the induction of circadian rhythms in clock gene expression in fibroblasts *in vitro* did not have any relation to the proliferative activity in general.

It has been found that the cycling of cryptochromes appear not necessary for circadian clock functions in mouse fibroblasts [205], challenging the view of a transcriptional-translational feedback loop in which the cycling of the CRY1 and CRY2 is thought to be necessary (Fig. 4). Hence, there may be a certain redundancy in the factors participating in circadian cycling, or there are individual differences between various differentiated cell types [206].

## The plant circadian clock

### Background

Circadian components in important processes as flowering and other daylength-dependent physiological phenomena were early recognized [207]. Circadian rhythms in CO<sub>2</sub> exchange [208, 209], enzyme activities, and transcript levels were since reported (for reviews see [10, 210, 211]). Recently, circadian rhythms in chromatin structure were observed in plants [212].

One of the most extensively studied gene families in plants, the *CAB* genes (*CHLOROPHYLL A/B-BINDING PROTEIN*), was shown to be expressed in a circadian manner, and also to be induced by light in many different plants including the model plant *Arabidopsis* [213-216]. These genes are encoded in the nucleus, translated in the cytosol, and then the proteins are imported into the chloroplasts to become components of the photosynthesis apparatus. Based on the properties of the *CAB* promoter, a pioneering method for picking clock mutants was developed [217]. A fragment of the *CAB* promoter, which was essential for light and circadian expression, was coupled to a luciferase reporter gene, and transformed into *Arabidopsis*. These transgenic *Arabidopsis* lines were then used to select for mutants in *CAB* rhythms recorded by fluorescence. A short period mutant, *toc1* (*timing of cab 1*), was identified and further characterized. In *toc1* plants the fluorescence rhythm linked to the *CAB* promoter was shortened to 20.9 h, whereas control plants showed a period length of 24.7. The rhythm in leaf movement also showed a shorter period (23.3 h) in the *toc1* mutant, compared with control plants (25.2 h). The *TOC1* gene was later cloned, and identified [218] as a gene encoding a PPR protein (pseudoresponse regulator protein). *TOC1* (or *PPR1*) is member of a small gene family in plants, comprising *PPR1*, *PPR3*, *PPR5*, *PPR7*, and *PPR9*, with partly overlapping functions. These proteins are reminiscent of the prokaryotic two-component kinases. They have a receiver domain containing a histidine, but the phospho-accepting aspartate residue present in prokaryotic two-components kinases is absent, suggesting that they do not function as the usual phospho-transfer proteins [219]. Further investigations showed that all five *PPR* genes were important for the clock functions [220, 221].

### *TOC1, LHY and CCA1 are essential elements in a plant clock mechanism*

It is now well established that expression of *TOC1* is influenced by a feedback loop comprising two closely related MYB factors *CCA1* (CIRCADIAN CLOCK ASSOCIATED 1) and *LHY* (LATE ELONGATED HYPOCOTYL) in addition to *TOC1* itself [222]. In this loop *TOC1* acts as a positive regulator of *CCA1* and *LHY* expression, whereas *CCA1* and *LHY* act to inhibit *TOC1* expression. *CCA1* and *LHY* bind to the promoter of *TOC1*, and thereby repress transcription of *TOC1*. The mechanism by which *TOC1* promotes expression of *CCA1* and *LHY* is not clear, but probably involves another protein, *PIF3* (PHYTOCHROME INTERACTING FACTOR) [223, 224]. The mutual influence of *TOC1* and *CCA1/LHY* have been well established through mutants, double mutants, and over-expressors. The results all support a regulatory model consistent with the positive and negative components of a feedback loop (Fig. 5) (for reviews see: [212, 220, 225, 226]). However, *TOC1* alone cannot induce expression of *CCA1* and *LHY*. Other genes are also necessary, i.e. *GI*, *ELF4* and *LUX*. The number of genes known to be related to the *TOC-CCA1/LHY* feedback loop is increasing, and a list of 20 genes was presented in a recent review by McClung [220].

Cryptochromes are the only conserved genes that appear to be commonly involved in eukaryotic clocks, i.e. in *Drosophila*, mammals and plants [227]. In plants, cryptochromes among other photoreceptors, are important for light-input to the clock. However, as for mammals, the cryptochromes are not essential for the plant core clock mechanism because in the *cry1 cry2* double knockout *CAB* expression was still circadian, although the period length was extended [228].

Changes in chromatin structure, are another emerging common feature of eukaryotic clocks. Recently circadian chromatin changes were also found in plants. Chromatin immunoprecipitation (ChiP) assays were performed with an antiacetylated Histones 3 antibody ( $\alpha$ ACH3), and subsequent PCR analysis of the *TOC1* promoter [229]. The results showed that histones bound to the *TOC1* promoter were acetylated in a circadian manner. The FACT (facilitates chromatin transcription) complex was also found to bind to the *TOC1* promoter in a circadian manner, further confirming the chromatin remodeling in parallel with *TOC1* expression [229].

Recently it has also been shown for *Arabidopsis* that phosphorylation and degradation of the TOC1 protein is important for clock function [221].

The balancing hypothesis for temperature compensation (see above) is supported by experiments showing that temperature compensation is achieved due to a dynamic balance between the genes *GI* and *LHY* [230]. These findings have also been confirmed by numerical simulations using an interlocking-loop model [231, 232] showing that balancing *LHY* against *GI* and other evening-expressed genes can largely account for temperature compensation in wild-type plants and the temperature-specific phenotypes of *GI* mutants.

## Conclusion

Circadian oscillators have evolved to adapt organisms to our planet's day/night cycles and to anticipate and meet unfavorable seasons. The core circadian oscillators are based on transcriptional-translational negative feedback loops and we are starting to understand and model the behaviors of the main molecular players within these oscillators and environmental influences. While transcriptional-translational negative feedback loops together with certain kinases and phosphatases appear to be conserved control structures among different organisms, the clock proteins are much more diverse and appear to have evolved independently.

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## Figure Legends

### Figure 1.

Schematic representation of a molecular mechanism for circadian oscillations with negative and positive feedback loops. Positive components/transcription factors interact with the promoter regions of clock genes leading to their expression and forming corresponding mRNAs and proteins. Some clock gene activation mechanisms may involve positive feedback loops. As supported by model calculations [40, 42, 43], the crucial element for getting oscillations is presence of one (or several) negative feedback loop(s), in which a clock protein inhibits its own transcription. Environmental influences affect the clock mechanism through a series of receptors which alter properties of clock proteins and their transcription factors through kinases and phosphatases, where some of phosphorylation and dephosphorylation pathways appear to be mechanistically conserved [69].

### Figure 2.

Growth tubes monitoring the free-running circadian rhythm in *Neurospora*. The sterile tubes contain growth medium (agar) and are sealed on each side with cotton plugs allowing air exchange. Inoculation with mycelium or conidia occur at one side of the tube. Under free-running conditions, generally in darkness or under a red safety light, the mycelium then grows along the tube with approximately constant speed [233]. Approximately every 22h conidia are formed seen as the patches on the tube reflecting the output of the circadian clock. The period of the free-running rhythm can be determined by measuring the distance between the conidial patches and dividing this distance by the growth speed.

### Figure 3.

Scheme of the circadian core network in *Neurospora crassa*. Several negative feedback loops have been identified. The FRQ protein plays a central role. Its highly regulated stability defines period length and temperature compensation of the conidiation rhythm [63, 64]. Additional feedback loops are also indicated. They seem to serve special purposes, for example when nitrate ion is the only source for nitrogen, or, as in the case of VIVID (VVD), playing a role in the phasing of the rhythm.

### Figure 4.

Model of the circadian core network in mammals. The heterodimer CLOCK/BMAL activates genes containing an E-box. CRY, the PER proteins and REV-ERB $\alpha$  are negative elements, while the ROR proteins together with CLOCK and BMAL1 define positive elements. For a more detailed discussion, see main text.

### Figure 5.

Feedback loops of the plant circadian network. Three loops are presently considered, the dawn-phased CCA1/LHY containing loop, which negatively regulates TOC1, a morning-phased loop containing the PRR proteins inhibiting the formation of CCA1/LHY, and an evening-phased loop, probably through GIGANTEA (GI) activating TOC1.

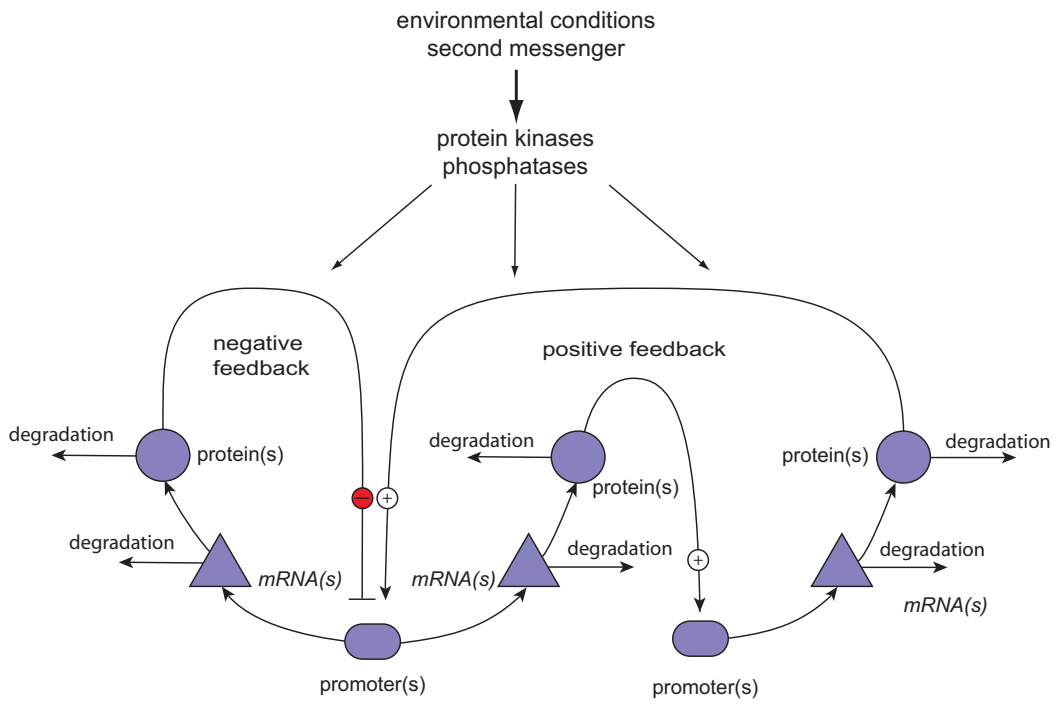


Fig. 1

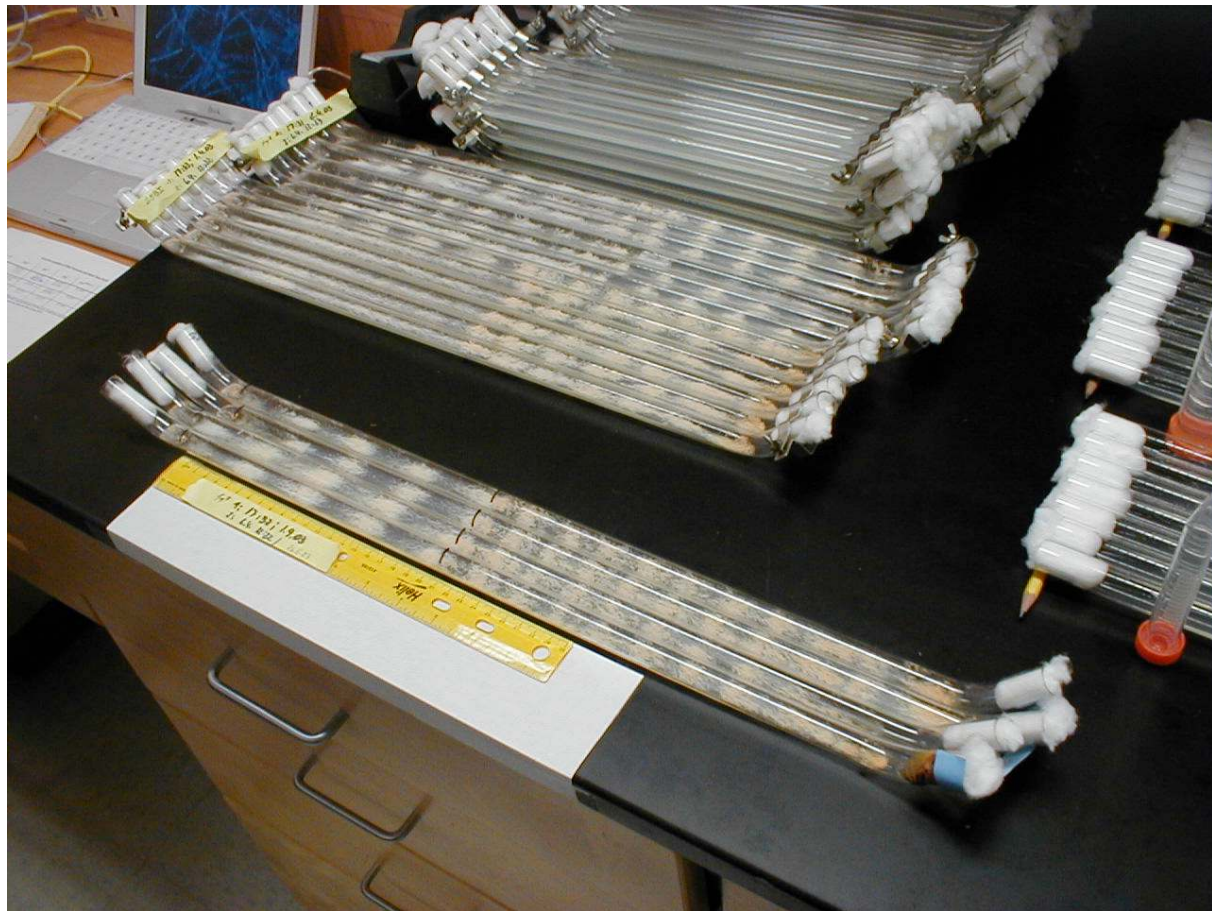


Fig. 2

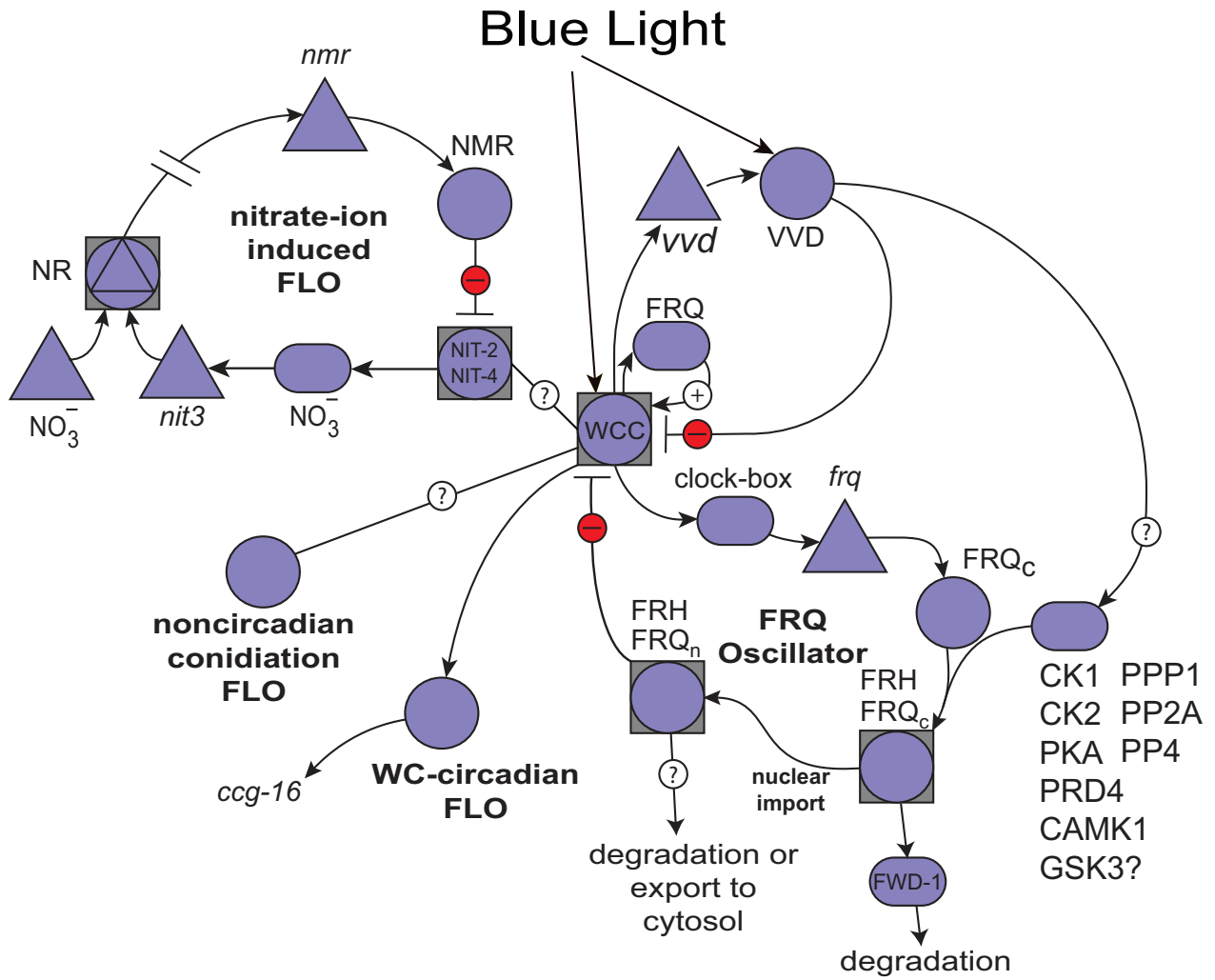


Fig. 3

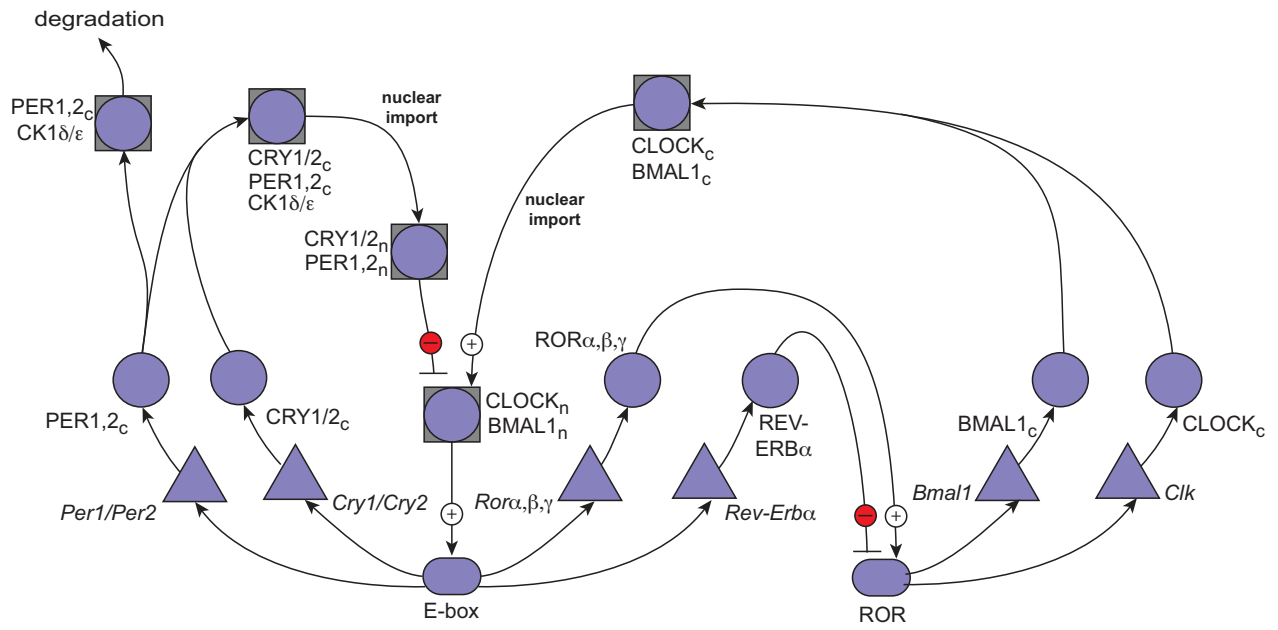


Fig.4

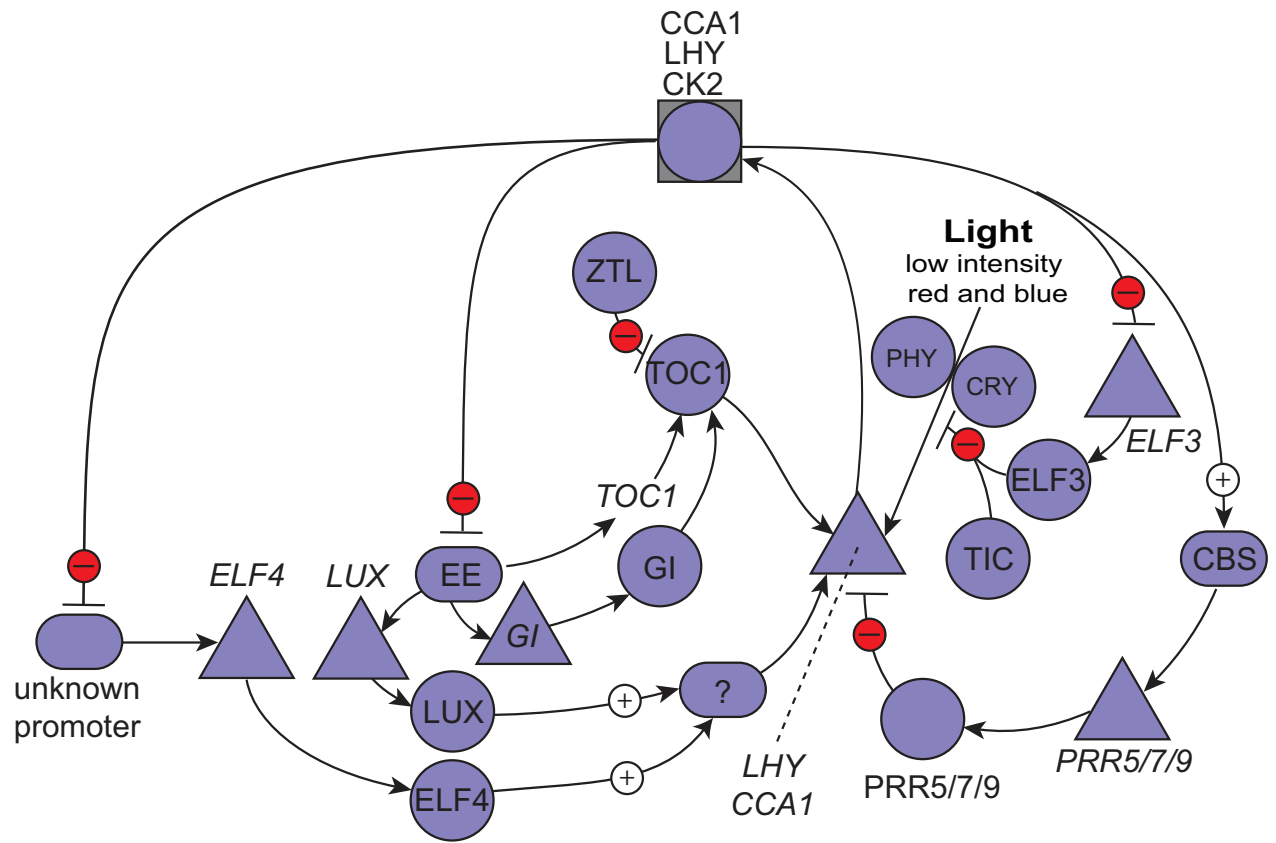


Fig.5