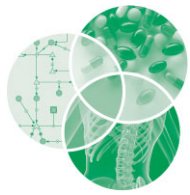
Advanced Review



Circadian oscillators in eukaryotes

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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 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 (TC) 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. © 2010 John Wiley & Sons, Inc. *WIREs Syst Biol Med*

Androsthenes 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 described 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] and by Linnaeus' famous 'flower clock' described in his *Philosophia Botanica*.[5] In the beginning of the 19th 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 have been the first person to work in the field that 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 1930s, Erwin Bünning suggested that intracellular time measurement leads to seasonal adaptations, such as flower induction, migration, and hibernation, which are based on an oscillatory and genetically determined physiological clock with a period of approximately one day. Although Bünning's hypothesis first caused major opposition, it became generally accepted during the 1950s.[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: (1) being endogenously generated; (2) showing a free-running rhythm; (3) can be phase-shifted by environmental perturbations, e.g., by light, temperature, chemicals; (4) they show entrainment, i.e., circadian oscillators can track rhythmic environmental changes; and (5) showing temperature compensation (TC), meaning that the free-running period is (approximately) the same at different but constant temperatures.

Circadian rhythms are important for the daily and seasonal adaptations 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 involves not only 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

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emphasis on the model organisms *Neurospora crassa, Arabidopsis thaliana*, and the mammalian clock. *Drosophila,* while a major model system, is left out here because of space limitations.

GENETICS AND MODEL ORGANISMS

In the beginning of the 1970s,[17] the first successfully generated clock mutants were generated with the fruit fly *Drosophila melanogaster*[18] and the filamentous fungus *N. 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 (Figure 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.[41–43] Kinetic models of transcriptional–translational negative feedback loops, some based on Goodwin's equations,[37,44] showed that many aspects of circadian oscillations including TC and phase resetting can be described.[38,39,42,45–60] Early predictions using the Goodwin oscillator indicated[38,61] that clock protein stability/turnover should determine the circadian period length, where short-period mutants should have a clock protein that is more rapidly turned over compared with wild type, whereas 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.[62–65] When certain phosphorylation sites in FRQ were blocked (i.e., replacing Ser 314 by an Ile),[63] FRQ stability increases and leads, as theoretically predicted,[38,61] to larger period lengths. In several follow-up papers by the Liu group,[66–68] it was found that phosphorylated FRQ is turned over by the ubiquitin–proteasome pathway.[69] The study of FRQ-decay kinetics in *Neurospora* clock mutants confirmed the theoretically predicted period–stability relationship with an intimate link to TC.[54,65] 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.[27,28,40,70–72] Similar observations have also been made for mammalian systems showing that the decreased period for the CK1ε *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 casein kinases (CK1 and CK2) and the phosphatase PP2A.[40]

Positive feedback loops (Figure 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 tunability of the oscillator,[79] whereas in other cases[80,81] the presence of an additional positive feedback did not seem to affect the robustness of the oscillator. In the case of the *Drosophila* oscillator, which at present includes two negative and one positive feedback loops, the positive loop is necessary to describe the influence of dosage of the *per*- and *vri*-genes 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 can even show TC.[93,98,99]

THE ISSUE OF TEMPERATURE COMPENSATION

Temperature compensation is one of the defining clock properties of circadian rhythms. TC means that the circadian period is homeostatically regulated toward variations in temperature, i.e., the circadian period is constant at different (constant) temperatures. TC is only operative within a certain organism-important temperature range. For most of the circadian oscillators the precise mechanism of how TC is achieved is still not known. A variety of suggestions how TC may be achieved have been considered during the years.[42,52]

In the 'balancing/opposing reaction approach', first suggested in 1957,[100] and later kinetically formulated for chemical oscillators,[55] each temperature-induced change in a rate constant of a reaction step

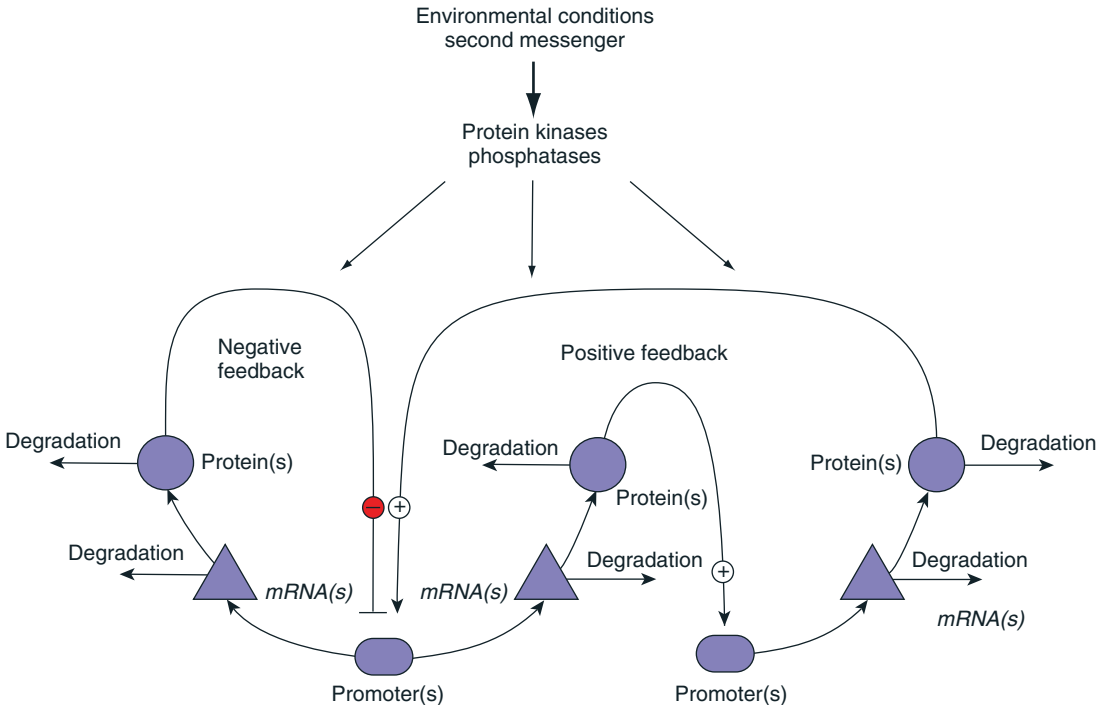

FIGURE 1 | Schematic representation of a molecular mechanism for circadian oscillations with negative and positive feedback loops. Positive components/transcription factors interact with the promoter regions of clock genes leading to their expression and forming corresponding mRNAs and proteins. Some clock gene activation mechanisms may involve positive feedback loops. As supported by model calculations,[37–39] the crucial element for getting oscillations is the 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 the properties of clock proteins and their transcription factors through kinases and phosphatases, where some of phosphorylation and dephosphorylation pathways appear to be mechanistically conserved.[40]

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.[52,101,102] This approach allows one to describe TC of any systemic property that depends on the rate constants, such as for nonoscillatory steady-state fluxes or steady-state concentrations,[51] 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 TC 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 TC. 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 cycle and a stable limit cycle. Indeed, there appears to be a close relationship between robust homeostasis and TC,[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.[56]

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.[27,28,40,70,108–111] 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 (Figure 2). A firefly luciferase-based reporter assay was first constructed by Morgan

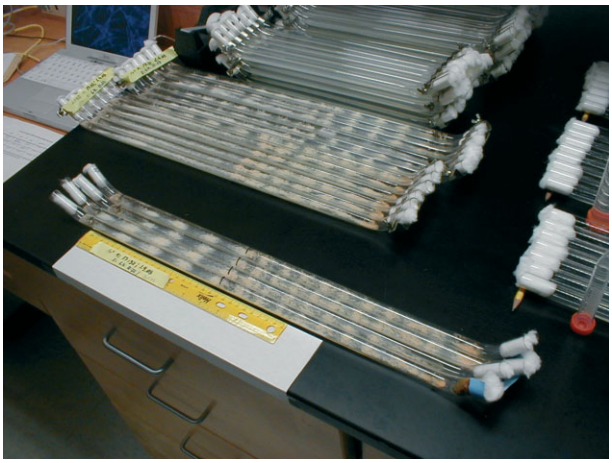

FIGURE 2 | Growth tubes monitoring the free-running circadian rhythm in *Neurospora*. The sterile tubes contain growth medium (agar) and are sealed on each side with cotton plugs allowing air exchange. Inoculation with mycelium or conidia occurs at one side of the tube. Under free-running conditions, generally in darkness or in a red safety light, the mycelium then grows along the tube with approximately constant speed.[116] Approximately every 22 h conidia are formed shown 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.

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 Section on FRQ-Independent Oscillators).

The basic mechanism behind the conidiation rhythm is a transcriptional–translational negative feedback loop, where the FRQ-protein inhibits its own transcription (FRQ-oscillator, Figure 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,[107,117–125] with WC-1 as a flavin-binding blue-light photoreceptor. The *frq* promoter contains two light responsive elements (LREs), where the distral element ('clock (C)-box')[126] 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.[127] Hong et al.[128] showed by model calculations that the binding of WCC to the *frq*-promoter is of importance for maintaining TC. 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.[129] Recent experimental evidence suggests that FRQ is rapidly shuttled between the nucleus and the cytoplasma,[130] which may be part of a FRQ-mediated mechanism to clear WC-1 out of the nucleus.

Although 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,[121,131,132] recent ChIP experiments indicate differential binding affinities of WC-1, WC-2 toward the LREs and a breakup of the WCC.[133] It was found that WC-1 is always bound to both LREs, whereas 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.[133]

As a result of a temperature-regulated alternative splicing mechanism, the FRQ-protein is found in a long form (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 TC is observed.[134–136] A recent kinetic model by Akman et al.[137] describes the temperature-induced two FRQ isoforms and the associated TC not only for the *bd* mutant but also for frq^1, frq^7, and frq^{S513I} mutants.

As already mentioned, the expressed FRQ-protein (i.e., both s- and l-forms) is posttranslationally 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.[64] CK2 has been found to be a key regulator of TC in *Neurospora*.[94] The *chrono* and *period-3* mutations have been found to be within the β1- and α-subunits of CK2. Reducing the dose of these subunits significantly alters TC indicating that TC is due to a balancing of positive and negative contributions to the period.[138]

Besides regulating FRQ-protein stability by proteasomal degradation,[68] there is now evidence that *frq*-mRNA is regulated by the exosome and defines an additional posttranscriptional negative feedback loop.[139]

FRQ dimerizes by a coiled-coil domain, which is important for maintaining circadian rhythmicity.[140] FRQ also binds to a 'FRQ-interacting RNA helicase', FRH.[141] Downregulation of FRH using RNA interference has been found to lead to increased *frq*-mRNA

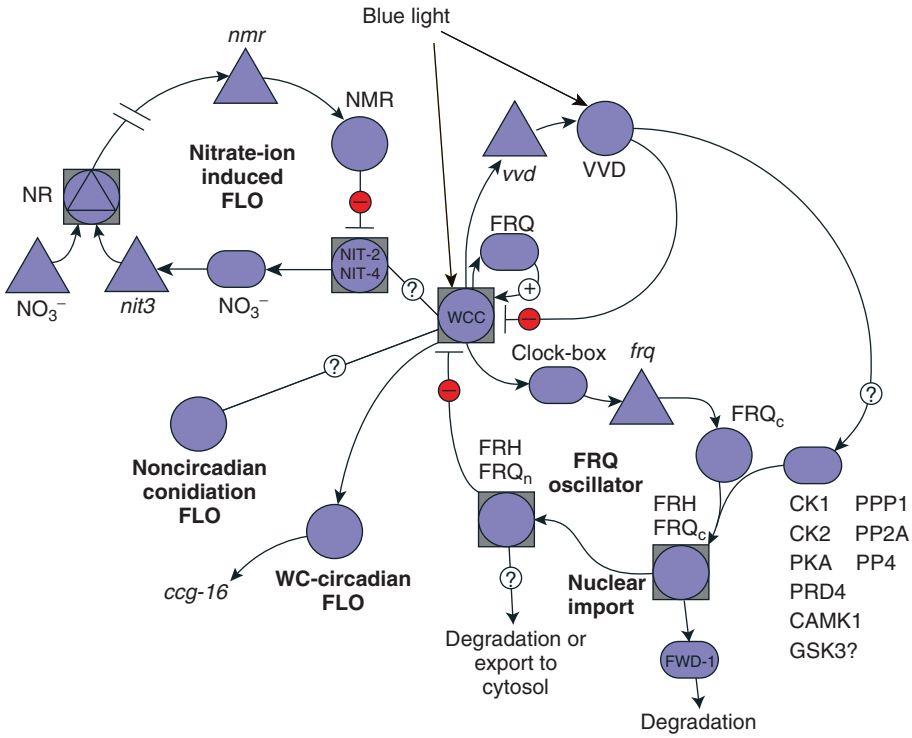

FIGURE 3 | Scheme of the circadian core network in *Neurospora crassa*. Several negative feedback loops have been identified. The FREQUENCY (FRQ) protein plays a central role. Its highly regulated stability defines period length and TC of the conidiation rhythm.[64,65] Additional feedback loops are also indicated. They seem to serve special purposes, i.e., 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.

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.[121,142,143] 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.[144] VIVID (VVD) is another light-upregulated and light-responsive protein, which contains a blue-light receptor.[145–147] The role of VVD is associated with the control of the phase of *Neuropsora's* circadian rhythm, its light resetting and transient light response[145,148–150] as well as the TC of the circadian phase.[151] In the vvd^{KO}, the phosphorylation pattern of FRQ is altered. At DD4, more of the lower-phosphorylated forms are seen in vvd^{KO}, whereas in the wild-type strain FRQ is hyperphosphorylated[151] indicating that VVD somehow interacts with FRQ and/or FRQ-phosphorylating or dephosphorylating processes. Schneider et al.[152] have recently found that a *vvd* mutant strain can show rhythmic conidiation under constant light (LL) conditions. The period of this strain ranges between 6 and 21 h 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.,[152] therefore concluded 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).[153,154] The first strain containing a FLO, frq^9, was characterized by Loros et al.[155] In this strain, a complete loss in TC 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^{10}).[156]

Several FLOs have now been identified, and alternative hypotheses for the 'circadian pacemaker' in *Neurospora* have been put forward.[152,153,157–161] 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.[162–164]

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 and 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 h.[33] These oscillations do not require a functional FRQ, but do require WC-1, and are observed both in darkness and 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.[165,166] 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 to act not only 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 nonimage photoreception and transmits this signal to the SCN. This is mediated by the pigment melanopsin,[167] which is accepted as a major component in the synchronization of circadian clocks.

The SCN has efferents to peripheral tissues, which constitute 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 tissues. 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.[168] 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-α) 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.[169,170]

The Cellular Clockwork

There is now increasing evidence that clock genes are expressed in the oocyte and during early embryonic development.[171] The mammalian circadian clock is a complex autoregulatory transcriptional and translational feedback program, which is composed of positive and negative regulators.[172] 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 (Figure 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.[173] In intact animals, transcription of *Per1* starts before dawn and has a peak in *Per1*-mRNA about 6 h later. The levels then rapidly subside before the end of the day. The resulting peak of the PER1 protein comes 6 h after its mRNA. *Per3* transcripts accumulate at the beginning of the day and subside after 4–6 h, whereas *Per2* mRNA accumulation occurs later than the other two genes and peaks at dusk. The transcripts of *Cry1* and *Cry2* reach a peak at 6–8 h after dawn and thereafter decline. In contrast to the transcripts, all the resulting proteins 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 (CK1ε/δ). It has been found that phosphorylation by CK1ε/δ is temperature-insensitive and period-determining,[174] probably by an 'instantaneous'[52] TC 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.[172] It has recently been found that CLOCK possesses intrinsic histone acetyltransferase activity in mouse liver cells, which contributes to chromatin-remodeling 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 downregulation of circadian transcription in clock-controlled genes.[175]

At least two other proteins may modulate PER1 activity in mammalian cells by regulating the circadian periodicity.[176] In addition, *Rev-erb-*α modulates the clock by prolonging the periodicity and also coordinating metabolic pathways.[177] Light then resets the master clock in the SCN, where the pigment melanopsin plays a central role.[167] However, the effect depends on the time when it acts, causing both phase

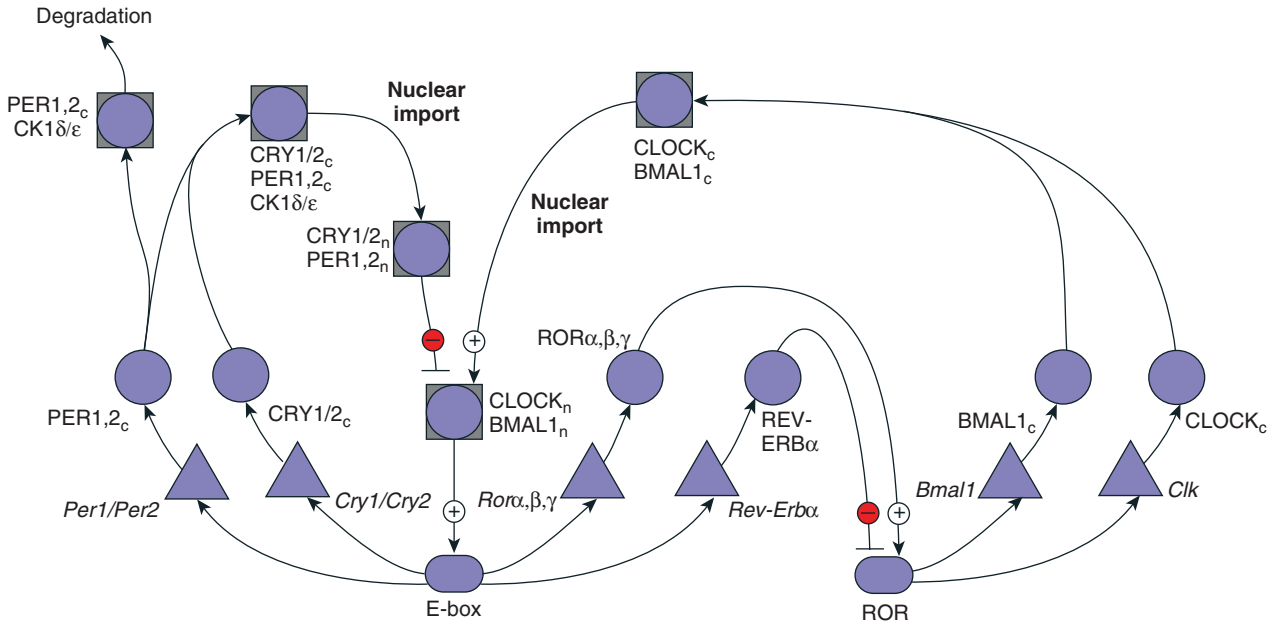

FIGURE 4 | Model of the circadian core network in mammals. The heterodimer CLOCK/BMAL activates genes containing an E-box. CRY, the PER proteins, and REV-ERBα are negative elements, whereas the ROR proteins together with CLOCK and BMAL1 define positive elements. For a more detailed discussion, see main text.

shift and modulation of the circadian phase.[167,178] In addition, at least two different types of mRNA exist that are interacting with the CLOCK–BMAL1 complex, whereby the circadian period is lengthened and the entrainment of the master clock by light is attenuated.[177]

Peripheral Clocks

The cloning and characterization of mammalian clock genes have revealed that they are generally expressed in a circadian manner in almost all organs of the body.[179] 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.[180] Still, it is not clear what causes the phase delay in some tissues. On the other hand, the rhythms of body temperature in rodents can sustain peripheral circadian clocks, being an indirect mechanism for phase synchronization.[181] Peripheral clocks also appear to be important for the regulation of cardiovascular and metabolic functions.[182]

Since 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.[183–188] It has been postulated that the whole immune system is both exogenously regulated and controlled by the endogenous clock from SCN.[189] In particular, BMAL1 seems to be important for the development of β-cells along a circadian time scale.[190] 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.[191]

Stem Cells

Several years ago, it was shown that the clonability of murine progenitor cells underwent circadian variations when cultured in semisolid medium.[192–195] 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[196] but also appeared to be developmentally regulated.[197] Subsequent sampling of human stem and progenitor cells (CD34+) from the bone marrow showed a different pattern, both with regard to phasing and amplitude.[198] Maximum mRNA level for *Per1, Per2*, and *Cry2*

was 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.[199] In line with this, it was reported that hemopoietic stem cell release in mice is regulated through circadian oscillations, peaking at 5 h after the initiation of light, and reaching a nadir at 5 h after darkness.[200]

Cultured human mesenchymal stem cells from the bone marrow can show circadian rhythms using serum shock[201–203] and cAMP analogs. The phosphorylation status of both PER1 and GSK3β was essential for getting circadian rhythms.[204] 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[205] It was shown that serum shock induced the circadian expression of various clock genes both in fibroblasts and in hepatoma cells from rats.[203] Later, it was shown that cAMP, protein kinase C, glucocorticoid hormones, and Ca^{2+} had the same effect.[201] Surprisingly, it was found that multiple signaling pathways in the cells could elicit circadian gene expression.[202]

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,[206] challenging the view of a transcriptional–translational feedback loop in which the cycling of the CRY1 and CRY2 is thought to be necessary (Figure 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.[207]

THE PLANT CIRCADIAN CLOCK

Background

Circadian components in important processes as flowering and other day length-dependent physiological phenomena were early recognized.[208] Circadian rhythms in CO_2 exchange,[209,210] enzyme activities, and transcript levels were since reported.[10,211,212] Recently, circadian rhythms in chromatin structure were observed in plants.[213]

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*.[214–217] 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.[218] 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 h. 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[219] 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-component kinases is absent, suggesting that they do not function as the usual phospho-transfer proteins.[220] Further investigations showed that all the five *PPR* genes were important for the clock functions.[221,222]

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 CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) in addition to TOC1 itself.[223] 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

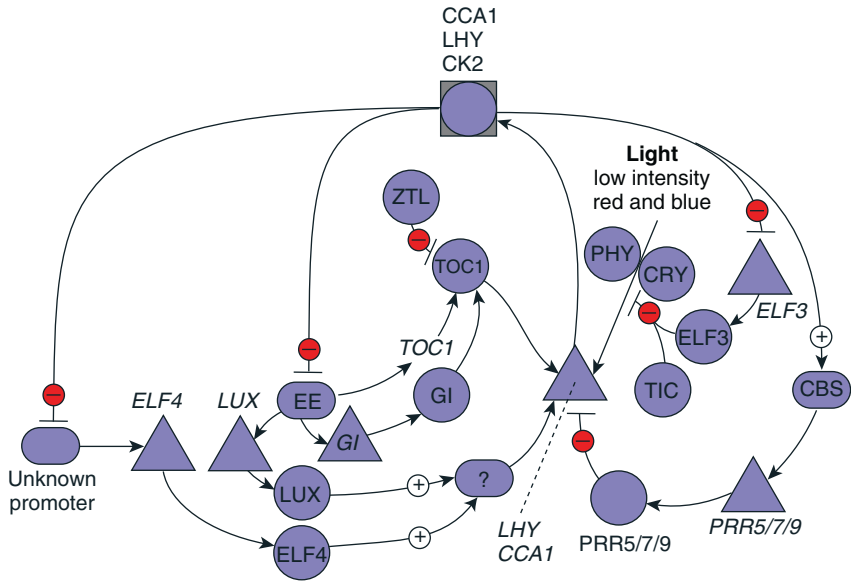

FIGURE 5 | Feedback loops of the plant circadian network. Three loops are presently considered, the dawn-phased CCA1/LHY containing loop, which negatively regulates TOC1, a morning-phased loop containing the PRR proteins inhibiting the formation of CCA1/LHY, and an evening-phased loop, probably through GIGANTEA (GI) activating TOC1.

repress transcription of *TOC1*. The mechanism by which TOC1 promotes expression of CCA1 and LHY is not clear, but probably involves another protein, PHYTOCHROME INTERACTING FACTOR (PIF3).[224,225] The mutual influence of *TOC1* and *CCA1/LHY* has been well established through mutants, double mutants, and overexpressors. The results all support a regulatory model consistent with the positive and negative components of a feedback loop (Figure 5).[213,221,226,227] However, *TOC1* alone cannot induce the 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.[221]

Cryptochromes are the only conserved genes that appear to be commonly involved in eukaryotic clocks, i.e., in *Drosophila*, mammals, and plants.[228] 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.[229]

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 (αACH3), and subsequent polymerase chain reaction (PCR) analysis of the *TOC1* promoter.[230] The results showed that histones bound to the *TOC1* promoter were acetylated in a circadian manner. The facilitates chromatin transcription (FACT) complex was also found to bind to the *TOC1* promoter in a circadian manner, further confirming the chromatin remodeling in parallel with *TOC1* expression.[230]

Recently, it has also been shown for *Arabidopsis* that phosphorylation and degradation of the TOC1 protein is important for clock function.[222]

The balancing hypothesis for TC (see above) is supported by experiments showing that TC is achieved because of a dynamic balance between the genes *GI* and *LHY*.[231] These findings have also been confirmed by numerical simulations using an interlocking-loop model[232,233] showing that balancing *LHY* against *GI* and other evening-expressed genes can largely account for TC in wild-type plants and for 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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