Circadian Rhythms

2. The Underlying Molecular Mechanisms

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Circadian (*circa* = about; *dian* = day) rhythms are amongst the most widely studied rhythmic behaviors across a wide range of organisms. Clocks driving circadian rhythms are termed circadian clocks and involve a network of functionally conserved genetic elements that mediate various oscillatory, physiological and behavioral phenomena and help tune the organism to remain in-sync with the external world. While some of the characteristic functional properties of circadian rhythms and clocks driving such rhythms have been studied for a long time now, our knowledge of the molecular and genetic architecture of circadian clocks remained elusive until recent years. This is the second article in a series on circadian rhythms and will introduce you to the molecular cogs and wheels of circadian clocks.

Circadian Clocks: An Introduction

Why is it that flowers bloom only at specific times of the day? Why is it that some organisms including human beings sleep at night and remain active during the day? Are these rhythmic phenomena mere responses to changing day and night conditions owing to the rotation of earth around its axis? Or is there an internal/physiological (endogenous) mechanism that drives them? Such questions have intrigued scientists for several decades and paved the way for the exploration of biological timing systems and their underlying mechanisms. Research on a wide variety of organisms has established that these rhythms are generated by internal time-keeping machinery called 'circadian clocks' which entrain to cyclic environmental cues called *zeitgebers* (German: *zeit* = time; *gebers* = givers) such as light, temperature or humidity and drive various behavioral and physiological rhythms so that their periodicities match the environmental factors. Under

constant conditions, i.e., in the absence of any zeitgeber, organisms continue to exhibit rhythmicity, albeit with near 24-h periodicities, indicating that these rhythms are generated from within the organism (see Part 1)[1]. Rhythms exhibited under constant conditions are termed 'free-running rhythms' and the period of such rhythms as 'free-running period (τ)' [1].

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A simplistic model of circadian clocks proposed that it comprises three functionally distinct components: (i) Input pathways – through which environmental time-cues such as light, temperature or humidity are perceived, (ii) Oscillator – an endogenous oscillation generating system which is capable of measuring the passage of time using near 24 h oscillation as a reference process, and (iii) Output pathways – that transduce the temporal information generated by the oscillator to various effecter organ(s)/ tissue(s) which regulate overt behaviour/physiological processes (*Figure* 1). In most cases, it is almost impossible to directly study the 'clock' and most rhythms studied (sleep/wake, body temperature, activity/rest cycles, rhythms in several physiological variables) are overt (output) rhythms and thus analogous to the hands of the clock. From the model of a circadian clock, it can be easily deduced that an aberrant overt rhythm does not necessarily imply a defective central clock, but can also be due to defects in the input pathways or the output pathways that represent the 'hands' of the clock while the underlying clock is still functional. So how does one go about testing how the central oscillator functions? This will be clear as we discuss some examples in later sections.

Our quest for understanding clocks has over the years revealed it to be a major component of our physiology. A range of physiological variables such as body temperature, hormone levels, blood pressure, psychomotor capabilities, perception of pain and

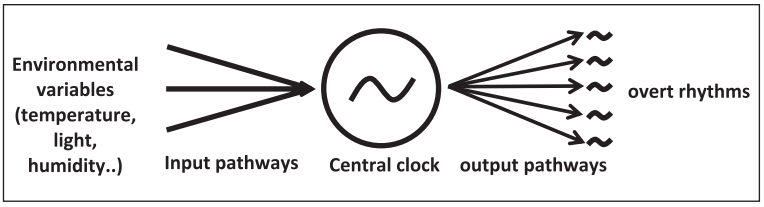

Figure 1. Pictorial representation of circadian clock organization.

Box 1.

Impaired circadian clock functioning has been shown to be associated with various sleep and mood related disorders. The Familial Advanced Sleep Phase Syndrome (FASPS) is a genetic disorder involving mutations in two circadian clock components: *period* and *casein kinase* genes. Subjects suffering from FASPS have advanced activity/rest rhythm and tend to go to bed about 3–4h earlier than others and also wake up early, which, in the long run affects their social life.

Seasonal Affective Disorder (SAD) is a class of mood disorder also referred to as 'winter blues' which involves recurrent depression occurring every year during the winters in otherwise healthy individuals living in temperate climates and is among the prominent causes for depression resulting in fatalities like suicides. SADs have been attributed to altered interaction between light and the circadian clocks. Also, subjects with mutations in the photoreceptors that convey light information to the clock show increased risk of SADs. Aberrant clocks have been associated with bipolar disorders and schizophrenia through impaired neurotransmitter release resulting from altered sleep patterns.

an array of metabolic processes exhibit circadian variation. Knowledge of circadian variation in these variables has had major implications in pharmacology – aiding designing and administering drugs to maximize therapeutic potential whilst minimizing side effects[2]. Also, it has been demonstrated that impairment or misalignment of circadian clocks with the external environment results in cardiovascular, metabolic, psychological and sleep disorders (*Box* 1) and thus, dissecting molecular mechanisms underlying circadian clocks can contribute to the development of effective therapeutic measures in future.

[2] Chemotherapy involves use of drugs that block DNA replication in malignant cells but end up affecting the normal cells as well. Recent studies have shown that DNA replication in cells exhibit circadian variation and thus subjecting cancer patients to chemotherapy at a time of the day when normal cells are less likely to be undergoing DNA synthesis can increase the effectiveness of treatment.

The Clockwork: Molecular Cogs and Gears of Circadian Clocks

Until as late as the 1900s, the notion that circadian rhythms are driven by endogenous mechanisms was not easily accepted and scientists studying biological clocks faced constant counter arguments from many eminent biologists claiming that circadian rhythms are responses to environmental cycles and not endogenous. While hindering the progress of the field for decades, this was also the driving force for studies that aimed at unequivocally establishing the endogenous and eventually genetic basis of circadian clocks.

Support in this direction came from several lines of study from

Box 2.

Jürgen Walther Ludwig Aschoff, born as fifth child of a pathologist Ludwig Aschoff and his wife Clara in Germany continued in the footsteps of his father and went on to pursue medicine before being appointed as Professor of Physiology at the University of Göttingen. His early work focused on homeothermy and thermal regulation which proved to be life changing and set Aschoff's professional career in a completely different direction. Experimenting on himself, Aschoff observed circadian rhythms in basal metabolic rate and body temperature, intrigued by which he went on to further pursue this feature. With the support of NATO, Aschoff set up the first isolation facility to study human circadian rhythms around the 1960s. Over 20 years of research in this facility helped Aschoff and his collaborator Rutger Wever to firmly establish that various aspects of human behavior, physiology and cognition were all clock controlled and that these discoveries had far reaching medical implications. Aschoff introduced much of the terminology in rhythm research, and the glossary of technical terminologies proposed by him is still being used. In honor of his outstanding contributions to the field of chronobiology, Aschoff's Rule Prize and Pittendrigh/Aschoff Lecture Prize is awarded to eminent researchers in chronobiology.

many researchers. Jürgen Aschoff (*Box* 2), a German physician, well known for his work on thermoregulation, raised mice for several generations in constant light and found that circadian rhythms continued to persist in the last generation as robustly as earlier despite the absence of cycling conditions for several generations thus indicating that circadian rhythms are an innate property of the organism [2]. Further, he also raised chicken in constant conditions and found that the adults continued to exhibit rhythmic behaviour, indicating that no prior exposure to cyclic conditions is required to develop rhythmicity [2]. While these studies demonstrated that circadian rhythms are not a learnt phenomenon or mere responses to light/dark cycles, another simple study by a German biologist Erwin Bünning[3] on bean plants in 1932 was the first step towards establishing the genetic basis of circadian clocks. Bünning, well known for his pioneering research in plant biology, made exceptional contributions to the development of the idea of phototropism and phototaxis. Bünning's work on circadian rhythms across model systems ranging from fungi to humans has helped lay the foundations for some of the conceptual underpinnings of chronobiology and he has been hailed one of the greatest biologists of the 19th century.

[3] See *Resonance*, Vol.1, No.7, 1996.

Bünning demonstrated that when pure bred short and long period

bean plants are interbred, the periods of their progeny are distributed around an average period value of both the parents indicating that properties of circadian rhythms are heritable and thus have a genetic basis [2]. Taking our understanding of the molecular genetic basis of clocks to the next step were studies on protists like *Euglena* and *Gonyaulax*. It was observed that the period of circadian rhythms can be modulated or the rhythm can be abolished by mRNA and protein synthesis inhibitors, implying that circadian clock mechanisms are encoded by the genome and require transcriptional and translational events to synthesize components of clock machinery. In spite of the growing evidence in favor of genetic basis of circadian rhythms, the underlying molecular mechanisms remained elusive due to the lack of advanced molecular genetic tools. The advent of mutagenesis screens[4] revolutionized the field of genetics and enabled discovery of genes controlling a plethora of behaviors and traits across organisms. Advancements in molecular biology tools and mutagenesis screens (*Figure* 2) led to a quantum leap in our understanding of

[4] Mutagenesis screens involves inducing random mutations in organisms by exposing them to mutagens (agents that induce mutation by altering the nucleotides in the genome), followed by screening for individuals showing aberrant phenotypes.

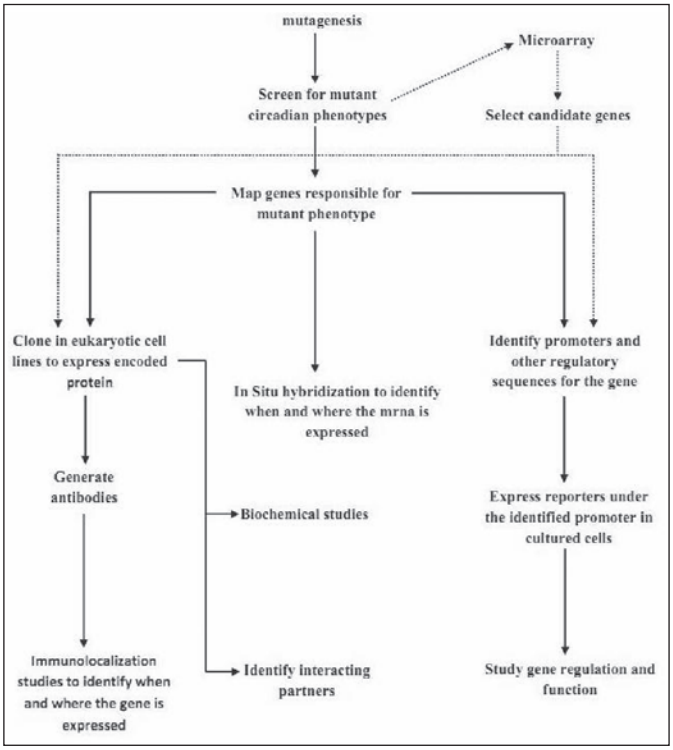

Figure 2. Mutagenesis screens have been instrumental in the identification and characterization of a majority of clock genes. The technique involves use of mutagens to induce mutations in the genome and screen for individuals with aberrant circadian phenotypes. The mutation is then mapped through several mapping strategies following which the genes are cloned, and the gene products are characterized. Various reporter genes (discussed later) are also used to study the upstream regulatory elements and gain insights into the mechanisms of circadian gene expression. Micro-arrays help in simultaneous assessment of expression of thousands of genes and have been used recently in the identification of clock genes as well.

the molecular genetics of circadian clocks by enabling discovery and characterization of several clock genes over the past 40 years.

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Discovery of the First Clock Mutant

A landmark paper published in 1971 provided unequivocal evidence in support of the genetic basis of circadian clocks and marked the beginning of the era of its molecular analyses. Ronald J Konopka working with Seymour Benzer (*Box* 3) at Caltech reported the discovery of the first ever clock gene in *Drosophila*. By subjecting flies to chemical mutagenesis and screening for flies displaying abnormal adult emergence rhythm, Konopka and Benzer isolated three mutants: (i) one exhibited a short τ of ~19 h, (ii) the second mutant had a long τ of ~28 h and (iii) the third one displayed arrhythmic adult emergence [3].

Even though these mutants were identified in a screen for aberrant adult emergence rhythm, they also exhibited similar patterns in activity/rest rhythm. Similar effects observed in two independent overt rhythms indicated that the mutation might possibly lie

Box 3.

Seymour Benzer pioneered the use of mutagenesis screens using *Drosophila* as a system to study various aspects of biology. He started his career as a graduate student of physics at Purdue University where his earlier work on Germanium formed the basis for the invention of the first transistor which won the Nobel Prize in Physics in 1956. Meanwhile, he developed an increasing interest in how genes regulate behaviour, possibly influenced by his two daughters who were quite different from each other in their behaviour even though reared and nurtured in the same environment. Initially he worked on genetics of T4 bacteriophage where he made prolific contributions to the genetics of phages. He is also well known for his work on elucidating codon degeneracy, a fundamental concept in molecular biology. Benzer used mutagenesis screens to isolate mutants with defective phototactic ability and observed that these mutants had defects in compound eye development. Further work in this direction led to the discovery of genes associated with development and cancer. He also developed assays to teach flies to learn and remember cues and eventually isolated mutants with defects in the ability to learn. His work has laid the cornerstone for further work in learning and memory in humans as well. He continued to use forward genetic screening to isolate and study mutants for variety of behaviours like locomotion, stress sensitivity, sexual function and muscle function, identifying several genes that affected behaviour thereby firmly establishing the notion that genes influence behaviour. From physics to biology and beyond, Benzer is one of the pioneering researchers of all time and is rightly known as the man who took us from genes to behaviour.

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in a gene/s which is a part of the common oscillator (*Figure* 1) that governs both the rhythms. Further, mapping revealed that the three mutations were alleles of the same gene on the X-chromosome and the gene was named *period* (*per*) since it affected the period of the rhythm. Mutations in the same gene rendering the clock fast or slow or even abolishing the rhythm further indicated that the *per* gene is indeed a component of the central oscillator.

This discovery had a huge impact on the field of chronobiology and the following decades saw growing interest in cloning and characterization of *per* gene, which was eventually accomplished 13 years later. The *per* mRNA and protein levels were found to exhibit circadian oscillation with their intracellular concentrations peaking at certain times of the day and reducing at other times. More importantly, the mRNA levels fell when the respective protein levels began to rise, hinting at a reciprocal relationship between the two.

Based on our understanding of physical oscillators (e.g., pendulum), much before the molecular mechanisms were deciphered, there had been long-standing speculation about the possible molecular architecture of circadian clocks. Any self-sustained oscillation requires the presence of positive and negative feedback loops built into the oscillator, which in turn determine the period of oscillation. Such oscillations had already been observed in metabolic cycles and almost all of these oscillations involve negative feedback mechanisms. Thus, feedback loops had already been implicated as an essential component of the circadian molecular clockwork. Further, based on the observation that *per* mRNA level falls as its protein levels peak, it was inferred that PER protein might act as a feedback regulator to suppress its own transcription (possibly by binding to its own promoter sequence). Studies also revealed that the PERIOD protein accumulates both in cytoplasm and nucleus at different times of the day indicating that PER probably shuttles between cytoplasm and the nucleus, further supporting the idea of a PER-mediated feedback loop. However, sequence analysis revealed

that PER proteins do not have any known DNA-binding domain[5] that could mediate binding of PER to the promoter sequences.

Another breakthrough was made in the year 1994 when a critical clock gene was identified, once again in a mutagenesis screen for aberrant emergence rhythm in *Drosophila* which, interestingly also exhibited activity/rest behaviours. As in the case of *per*, this mutation also resulted in similar changes in the two independent overt rhythms and eventually the gene was found to be a component of the central oscillator as well. The mutation was in a gene on the second chromosome and was named *timeless* (*tim*) with the null mutant being *tim* zero/null (tim^0) [3]. Like per^0, tim^0 mutation was found to affect both mRNA and protein oscillations suggesting that in addition to PER, TIM protein may also influence its own transcription and was later reported to physically interact with PER to form a PER–TIM dimer. This led to the speculation that TIM might have a DNA-binding domain that mediates binding to promoter sequences and represses *per* and *tim* expression, but TIM protein also lacked any known DNA-binding domain and thus, more interacting partners of PER–TIM dimer were expected to be involved in the feedback regulation. Nevertheless, TIM had more interesting properties that were unraveled later. When flashed with a pulse of light, TIM degraded within 30 min indicating that it is a photosensitive protein. Even though circadian clocks had already been known to be influenced by light, this discovery was particularly interesting because it was the first step towards unraveling the molecular mechanisms underlying the effect of light on circadian clocks.

[5] DNA-binding domains are protein domains that have the ability to recognize and bind to specific DNA sequences. Proteins containing DNA-binding domains are generally associated with DNA replication, repair or regulators of gene expression. Some of the well-known DNA binding domains include helix-turn-helix, Leucine zipper, helix-loop-helix and Zinc finger.

Closing the Feedback Loop

Meanwhile, another major discovery aided a great deal in the search for the interacting partners of PER–TIM dimer. In 1997, an enhancer region called E-box[6] was discovered upstream of *per* gene and was shown to be essential for the rhythmic transcription of *per* [3]. Thus, identification of transcription factors that bind to E-box would further help unravel the mechanism of transcriptional regulation of clock genes. This time, help arrived

[6] Enhancer Box (E-box) is a DNA sequence that lies in the promoter region of a gene. It has a consensus sequence CANNTG and is bound by transcription factors which have basic helix-loop-helix domains. E-box was also found to be a part of the regulatory sequence of *tim* and eventually several other clock controlled genes.

from the mammalian counterparts when a novel circadian rhythm mutant in mice exhibiting a longer clock period of 24.8 h was reported. The mutation was mapped and the locus was dubbed *mclock* (circadian locomotor activity output clock kaput). Following this discovery, a *clock* gene homologue of *mclock* was identified in *Drosophila* and named *dclock* (*Drosophila clock* [3]). In yet another mutant screen in *Drosophila*, a gene *cycle* that codes for the protein CYCLE (CYC) was identified which shared similarities with another mammalian counterpart BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator-like). Both CLK and CYC harbored the bHLH domain and were reported to form a hetero-dimer; bind to E-box and initiate *per* and *tim* transcription [3]. Thus, with a consecutive publication of reports in a span of 2 years, many missing links in the molecular clockwork were discovered and the negative feedback loop was finally deciphered (*Figure* 3). The advent of microarray technology and other parallel advances in molecular genetic analyses techniques led to the identification of several other genes which form components of an additional loop in the core clock. Since the purpose of this article is to introduce the readers to molecular clockwork regulating circadian rhythms, we will not discuss other additional loops here but a detailed discussion on this topic can be found elsewhere [3].

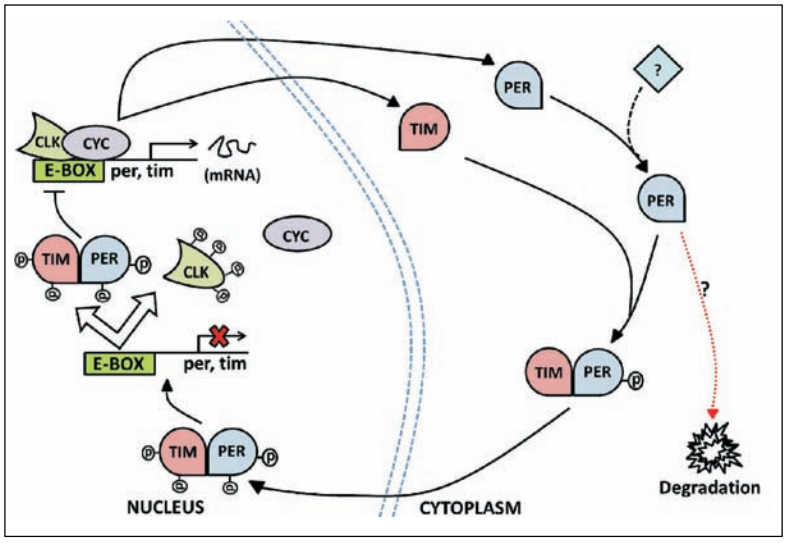

Figure 3. Graphical representation of molecular components of negative feedback loop. CLOCK (CLK) and CYCLE (CYC) form a hetero-dimer; bind to the E-box and transcribe *period* and *timeless* genes. While PER phosphorylation results in its degradation, binding of TIM to PER stabilizes PER and the PER–TIM complex then enters the nucleus where it displaces CLK–CYC hetero-dimer from the E-box thereby inhibiting transcription of *per* and *tim*. TIM undergoes degradation in response to light and is believed to mediate entrainment of circadian clocks to light/dark cycles.

Effect of Light on Circadian Clock

With the realization of the core feedback loop, the question of how light affects the molecular clocks continued to intrigue the field. Discovery of the photosensitive nature of TIM was a short-lived delight as sequence analysis revealed that TIM is not homologous to any known photoreceptor. Nevertheless, earlier studies had already revealed some important features regarding photosensitivity of the circadian system in *Drosophila*. The *Drosophila* circadian system is maximally sensitive to blue light and thus a blue light photoreceptor was long implicated to be involved in photosensitivity of circadian clocks. Flies reared on carotenoid[7] deficient diet did not show any defect in circadian entrainment to light/dark cycles. Also, mutants lacking external eyes and other components of visual transduction pathways showed minor defects in circadian entrainment or degradation of TIM in response to light indicating that the photosensitivity of circadian clocks in *Drosophila* was not solely mediated by carotenoid based photoreceptors. This indicated that there existed a novel photopigment which, in combination with the external eyes and other known photoreceptors, mediated the sensitivity of the circadian clock to light. This novel photopigment was finally discovered in another mutagenesis screen on *Drosophila.* The mutation affected the light sensitivity of circadian clock and was mapped to a gene whose sequence shared homology with a class of blue-light photoreceptors in plants called *cryptochrome*; thus the gene was dubbed *cry* [3]. In these mutants, TIM no longer degraded in response to light indicating that CRY is essential for light-induced degradation of TIM. CRY was later found to associate with TIM (only in the presence of light) and induce degradation of TIM, thus shedding light on how TIM degrades in response to light.

Apart from the central clock genes, genetic screens also led to the identification of kinases that play a major role in mediating stability and nuclear entry of PER and TIM. While a kinase, DOUBLETIME (DBT) was found to associate with PER and mark it for proteasomal degradation[8], TIM forms a PER–DBT–

The *Drosophila* circadian system is maximally sensitive to blue light and thus a blue light photoreceptor was long implicated to be involved in photosensitivity of circadian clocks.

[7] Carotenoids are organic pigments which are converted to aldehyde form of Vitamin A called retinals (Vitamin A Aldehyde). Opsins which are components of rods and cones (photoreceptors) associate with retinals and thus, carotenoid deficient diet leads to impaired photosensitivity in organisms due to defective rods and cones.

[8] Proteasomal or ubiquitin-proteasome degradation is the process by which a large protein complex called a proteasome degrades other proteins by proteolysis (lysis of peptide bonds). Phosphorylation of some proteins targets them for ubiquitination (attachment of a small regulatory protein, ubiquitin) by an enzyme ubiquitin ligase which are then recognized by proteasome and degraded.

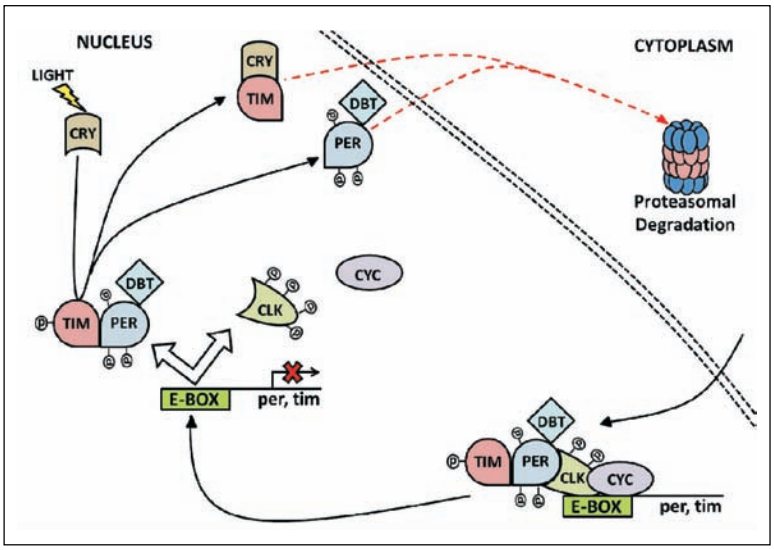

Figure 4. The figure depicts molecular events following the entry of PER–DBT–TIM complex into the nucleus leading to the repression of *per* and *tim* expression. Following nuclear entry, the PER-DBT-TIM complex binds the E-box bound CLK–CYC heterodimer to form a PER–DBT–TIM–CLK–CYC complex, and DBT also hyperphosphorylates CLK. CLK upon hyperphosphorylation, dissociates from the complex releasing the CLK–CYC heterodimer from E-box thereby repressing *per* and *tim* expression. In addition, lights during the day induces CRY mediated degradation of TIM, and with no TIM to stabilize, DBT bound PER is marked for proteasomal degradation.

TIM complex and stabilizes PER. CASEIN KINASE 2α (CK2α) and SHAGGY (SGG) kinases phosphorylate PER and TIM respectively in the PER–DBT–TIM complex promoting its nuclear localization. Once inside the nucleus, this complex displaces the CLK–CYC heterodimer from the E-box thus bringing about the repression of *per* and *tim* transcription (*Figure* 4) [3].

To summarize (*Figure* 5), the molecular components of the core circadian oscillator in *Drosophila* involves transcription factors CLOCK and CYCLE that form a CLK–CYC heterodimer, bind to E-box sequences on the *per* and *tim* genes and induce its transcription through the day until midnight when the mRNA levels of both genes peak.

Subsequently, PER and TIM proteins peak by early morning in the cytoplasm where DBT kinase associates with PER and promotes degradation of PER which is later stabilized by binding of TIM to PER–DBT complex. Subsequent phosphorylation by SGG kinase and CK2α promotes translocation of the PER–DBT–TIM complex to the nucleus where it displaces CLK–CYC heterodimer from the E-box thus repressing *per* and *tim* transcription and completing the feedback loop. In addition to this, when lights turn ON in the morning, TIM in the PER–DBT–TIM

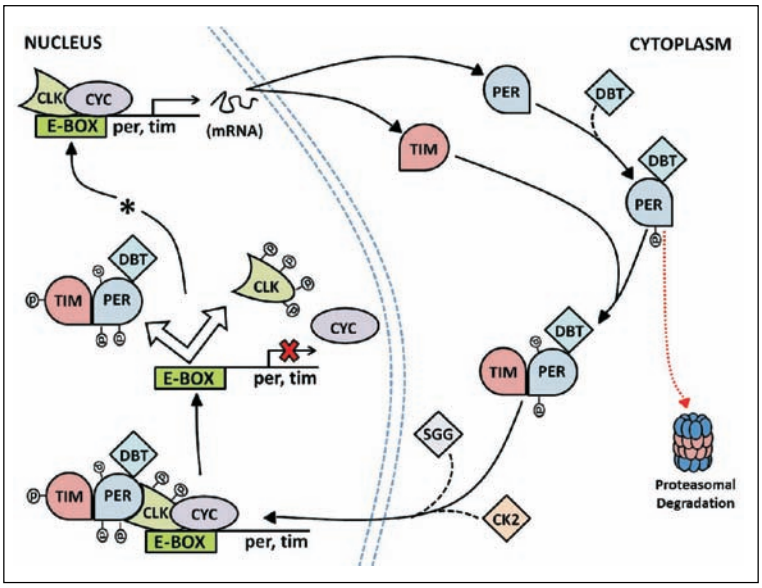

Figure 5. Pictorial representation of molecular components of the circadian clock. The CLK-CYC heterodimer binds to E-box and transcribes *period* and *timeless* which are then translated into respective proteins. Following phosphorylation by DOUBLETIME KINASE(DBT), PER is subjected to proteasomal degradation. Meanwhile TIM binds to PER and stabilizes the PER–DBT complex. Phosphorylation by SHAGGY KINASE (SGG) and CASEIN KINASE2 (CK2) promotes the nuclear localization of the PER–DBT–TIM complex where it inhibits transcription by CLK–CYC heterodimer thus forming a negative feedback loop spanning 24 h. Also, TIM undergoes proteasomal degradation by light and mediates entrainment of the circadian clocks to light/dark cycles.

complex undergoes CRY-mediated degradation leaving behind unstable PER–DBT complex. Due to lack of stability in the absence of TIM, PER is then targeted for proteasomal degradation by DBT. This set of events takes approximately 24 h to complete and constitutes one circadian cycle.

Even though our discussion drew insights mostly from components of *Drosophila* circadian clockwork, barring minute differences and details, the architecture and functional principles driving molecular clocks in all organisms ranging from algae to mammals remain quite similar and can be summarized in three take home messages:

1. At the molecular level, circadian clocks include a set of core clock genes whose expression exhibit oscillations over a period of 24 h and mutations in any of these genes affect the functionality of the circadian clock. These core clock genes generally form the components of a negative feedback loop essential for the generation of circadian rhythms.

2. These core clock genes are under post-transcriptional and post-translational regulation mostly mediated by kinases that influence the stability and movement of clock proteins from

the cytoplasm to the nucleus. Mutations in these kinases mostly affect clock period indicating that these mechanisms are essential to introduce time delays at various steps and eventually generate a 24-h cycle.

3. Circadian clocks are entrained by various cyclic environmental factors. All clocks have one or more photosensitive components that mediate interaction of light with the circadian clock to bring about entrainment. In addition to light, temperature and humidity serve as additional *zeitgebers* for circadian entrainment and are mediated by other mechanosensory receptors.

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Suggested Reading

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