Interactive Features of Proteins Composing Eukaryotic Circadian Clocks

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Abstract

Research into the molecular mechanisms of eukaryotic circadian clocks has proceeded at an electrifying pace. In this review, we discuss advances in our understanding of the structures of central molecular players in the timing oscillators of fungi, insects, and mammals. A series of clock protein structures demonstrate that the PAS (Per/Arnt/Sim) domain has been used with great variation to formulate the transcriptional activators and repressors of the clock. We discuss how posttranslational modifications and external cues, such as light, affect the conformation and function of core clock components. Recent breakthroughs have also revealed novel interactions among clock proteins and new partners that couple the clock to metabolic and developmental pathways. Overall, a picture of clock function has emerged wherein conserved motifs and structural platforms have been elaborated into a highly dynamic collection of interacting molecules that undergo orchestrated changes in chemical structure, conformational state, and partners.

Contents

POSTTRANSLATIONAL REGULATION OF CIRCADIAN RHYTHMS IN ANIMAL CELLS

Circadian clocks are often depicted as networks of transcription factors that regulate their own production at the level of RNA synthesis. For example, in *Drosophila*, two proteins, Period (PER) and Timeless (TIM), negatively regulate their own expression by suppressing two activators of *per* and *tim* transcription, dClock (dCLK) and Cycle (CYC) (**Figure 1**) (1, 2). Nevertheless, genetic and biochemical studies of circadian clocks in both prokaryotic and eukaryotic organisms have identified posttranslational controls that are essential to the production of molecular oscillations. Posttranslational modifications can specify longevity, activity, and the subcellular localization of proteins composing circadian clocks. Genetic screens have demonstrated that evolutionarily conserved, posttranslational controls determine the period length of the circadian oscillation and provide a basis for entrainment to environmental cycles.

Phosphorylation and Glycosylation Determine Clock Protein Function

Doubletime (*Dbt*), which encodes casein kinase 1 (CK1), was the first clock gene to be linked to posttranslational control of circadian rhythmicity. Discovered in *Drosophila*, *Dbt* phosphorylates the transcription factor PER (**Figure 1*a***). Loss of *Dbt* (or sharply reduced DBT function) produces behavioral and molecular arrhythmicity that was associated with increased PER stability and altered PER subcellular localization (3–5). Regulation of PER stability ultimately involves Slimb, an F-box/WD40-repeat protein that interacts preferentially with DBT-phosphorylated PER. This association with Slimb moves PER to the ubiquitin/proteasome pathway for degradation (6, 7). Nevertheless, before Slimb is recruited, DBT appears to phosphorylate PER at several sites in a stepwise fashion that encompasses several hours in wild-type *Drosophila* (8). DBT is physically associated with PER and TIM (see later in this section) (**Figure 1**) throughout much of this multihour interval, and the subcellular distribution of DBT in many tissues is dictated by that of PER and TIM at times of day when the latter proteins are abundant (9). This finding suggests that PER may also rhythmically affect the activity of DBT with respect to DBT's many alternative substrates (9).

Although some phosphorylations produced by DBT have been associated with decreased PER stability, others appear to affect PER's activity as a transcriptional repressor (7, 8, 10).

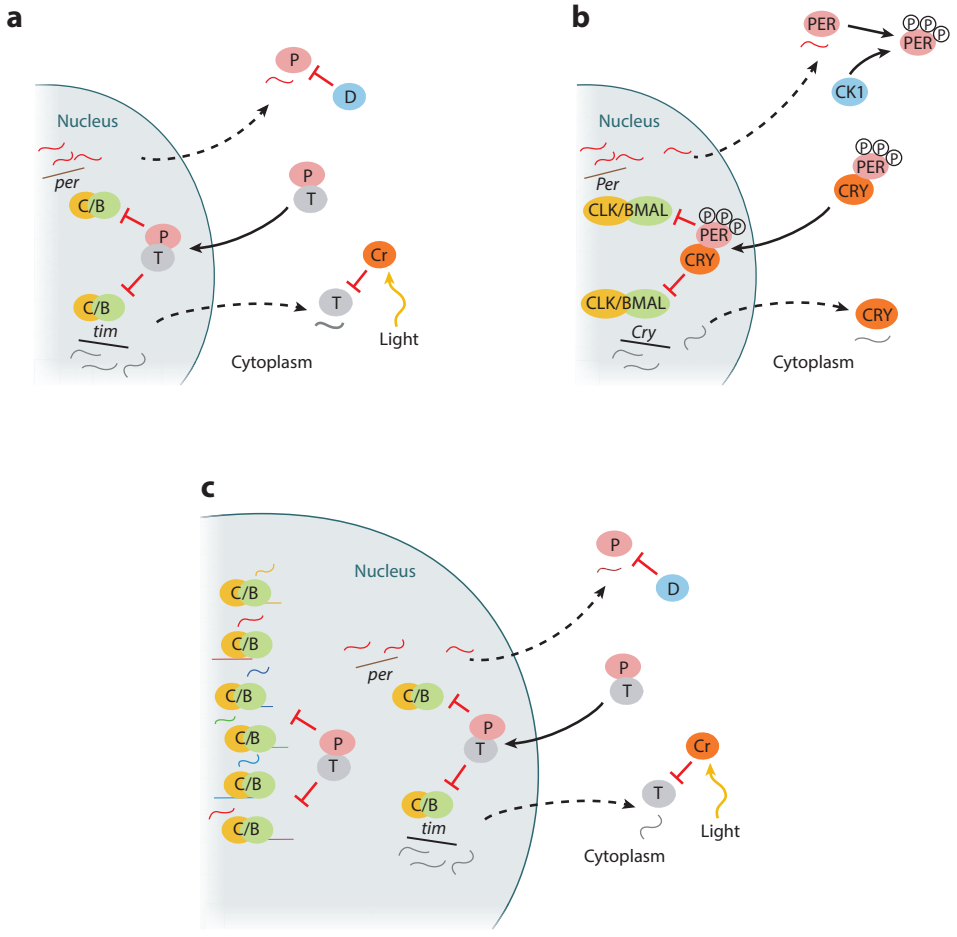

Figure 1

Principal elements of the circadian clocks of *Drosophila* and mammals. (*a*) In *Drosophila*, in the absence of nuclear Period (PER, P) and Timeless (TIM, T) proteins, transcription factors dClock (dCLK, C) and Cycle (CYC, B) drive *per* and *tim* gene expression. PER and TIM proteins physically associate in the cytoplasm, and after a delay of several hours both proteins accumulate in the nucleus. PER is phosphorylated and destabilized by Doubletime (DBT, D) in the absence of TIM, slowing down cytoplasmic accumulation of PER and PER/TIM complexes. Nuclear PER and TIM interact with dCLK/CYC complexes and block further transcription of *per* and *tim*. In the presence of light, the photoreceptor Cryptochrome (Cr) binds TIM, and both proteins are rapidly degraded. Such daylight-induced loss of TIM reinforces molecular rhythmicity. DBT-dependent nuclear phosphorylations of PER eventually trigger PER turnover and resumption of the circadian cycle. Dashed arrows indicate RNA movements; solid arrows indicate movement of proteins. (*b*) In mammals, CLOCK (CLK) and BMAL1 (BMAL) activate *Per* and *Cry* expression. Mammalian PER proteins are phosphorylated by casein kinase (CK)1δ and CK1ε (both orthologs of *Drosophila* DBT), which affects PER function, subcellular localization, and stability. Physical association between PER and CRY promotes nuclear accumulation of both proteins (19), and nuclear PER/CRY represses *Per* and *Cry* expression by suppressing the activity of CLOCK/BMAL1. (*c*) In *Drosophila*, rhythmic PER/TIM interactions with dCLK/CYC produce circadian cycles of activity for hundreds of clock-controlled genes (CCGs) (66, 231). Broad CCG programs are also found in mammals and influence almost every aspect of biology (232). This regulation is achieved through direct, cycling associations between CCGs and PER and other clock proteins, by secondary cascades of such regulation (233), and by physical interactions that couple clock proteins to certain kinases, nuclear receptors, and other regulatory factors (also see **Figure 3**).

Because DBT-dependent PER phosphorylation influences the duration of PER function, altered DBT kinase activity should affect the period of the circadian clock. Consistent with this view, mutations that lower DBT activity increase the period length of the circadian cycle (11, 12). CK1 similarly regulates the circadian clocks of mammals, including humans, through interactions with PER orthologs (e.g., References 13–22).

In wild-type *Drosophila*, PER is stabilized in the presence of DBT by physical interaction with a second transcription factor, TIM (**Figure 1*a***). This interaction between PER and TIM is also required for their nuclear accumulation and, thus, transcriptional autoregulation of *per* and *tim* gene activity (**Figure 1*a***) (23, 24). In mutants that lack TIM, PER fails to accumulate in cells expressing *per* mRNA (23). Mutant PER proteins that are poor DBT substrates, however, are stable in the absence of TIM and accumulate to high levels in the cytoplasm (2, 23, 25). These mutant proteins are transported to nuclei in the presence of TIM, indicating that DBT normally acts as a brake to cytoplasmic accumulation of PER in the absence of TIM (23, 25). Because DBT suppresses accumulation of cytoplasmic PER proteins that are not bound to TIM, the timing of PER–TIM interaction and nuclear accumulation is preceded, and therefore delayed, by DBT–PER interaction (3, 9). DBT also contributes to this delay by blocking nuclear accumulation of PER proteins that are not associated with TIM (**Figure 2**) (5).

This regulation has been further studied in TIM mutants that carry an altered nuclear localization sequence (NLS) and in TIM mutants bearing another variation that impedes TIM nuclear accumulation [TIM P116L (25), TIM-NLS (26)]. Wild-type PER proteins accumulate to high levels in the cytoplasm of *tim-NLS* mutants, but the nuclear accumulation of PER and TIM-NLS proteins is severely delayed. Flies producing wild-type PER proteins and TIM-NLS or TIM P116L proteins have long-period molecular and behavioral rhythms that are consistent with the altered accumulation profiles of PER and TIM-NLS (25, 26).

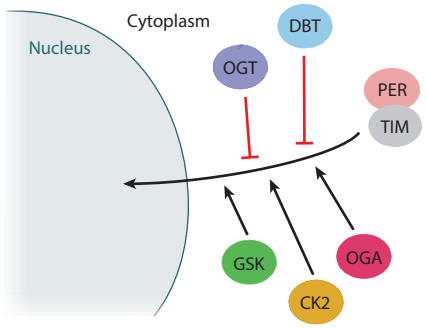

Figure 2

Control of Period–Timeless (PER–TIM) nuclear accumulation. In *Drosophila*, PER and TIM coexpression is required for nuclear accumulation of either protein. Nuclear accumulation is further regulated by glycosylation and phosphorylation of one or both proteins. *O*-GlcNAc transferase (OGT) adds *N*-acetylglucosamine residues to PER, and *O*-GlcNAcase (OGA) removes them. Increasing the level of OGT delays the timing of nuclear accumulation of PER and TIM. Phosphorylation of PER and/or TIM by glycogen synthase kinase 3 (GSK) and casein kinase 2α (CK2) promotes PER–TIM nuclear accumulation. Biochemical studies of PER in *Drosophila* and mammals indicate that glycosylation precedes phosphorylation and may block certain sites of PER phosphorylation, including sites that are targets of Doubletime/casein kinase 1 (DBT/CK1). In *Drosophila*, DBT inhibits nuclear accumulation of monomeric PER proteins (5).

Just as PER requires TIM for nuclear accumulation, mutations that eliminate PER cause aberrant cytoplasmic accumulation of TIM proteins (**Figure 1**) (27, 28). Also, recent studies have shown that regulated translation of PER may contribute a further delay mechanism (29, 30).

In addition to delays that may reflect translational controls, or changing concentrations of cytoplasmic PER and TIM during each circadian cycle, cytoplasmic PER/TIM complexes must be modified to permit nuclear accumulation. These modifications appear to contribute to a so-called interval timer that promotes cytoplasmic retention of PER/TIM complexes for several hours following their formation. Such delays have been studied in

flies and in cultured *Drosophila* cells (S2 cells). Perhaps the clearest indication that physical interaction between PER and TIM is not sufficient to trigger nuclear accumulation comes from fluorescence resonance energy transfer (FRET) assays of PER–TIM interactions in live S2 cells (31). FRET emerges rapidly if high levels of PER-CFP and TIM-YFP are induced, suggesting rapid formation of PER/TIM complexes in S2 cells. Nevertheless, the onset of nuclear accumulation of the tagged proteins followed the appearance of FRET by several hours (31, 32). The alternative expression of mutant proteins PER-L and TIM-NLS, both of which yield long-period circadian rhythms associated with a delay in nuclear accumulation of PER and TIM in adult *Drosophila*, further separated phases of FRET and of nuclear accumulation in S2 cells. This finding suggests that the long-period mutations affect a process (or processes) that is (are) preceded by the physical association between PER and TIM (26, 31).

Three kinases in addition to DBT (CK1) may influence this interval timer in *Drosophila* (**Figure 2**). Studies of *Drosophila* with mutant forms of glycogen synthase kinase 3 (GSK-3), and of *Drosophila* in which the dosage of GSK-3 has been altered, indicate that both PER and TIM may be phosphorylated by this kinase in a fashion that can advance the timing of nuclear accumulation (25, 33–35). Certain GSK-3-dependent phosphorylations of PER depend on prior PER phosphorylation by an unidentified proline-directed kinase (35). Thus, a proline-dependent kinase and GSK-3 may cooperate to establish the correct phase of PER nuclear accumulation. Finally, hypomorphic mutations of casein kinase 2α (CK2α) produce long-period circadian rhythms that are associated with delayed nuclear accumulation of PER and TIM (36–38). CK2 may play a related role in the mammalian circadian clock. CK2 overexpression and inhibition also shorten and lengthen circadian period, respectively, in cultured mammalian cells (39, 40).

Glycosylation (*O*-GlcNAcylation) regulates the timing of PER nuclear accumulation in *Drosophila* (**Figure 2**). *O*-GlcNAc transferase (OGT) adds *N*-acetylglucosamine to serine and/or threonine residues on PER. This regulation occurs with a distinct circadian rhythm that precedes PER hyperphosphorylation by DBT (41, 42). *O*-GlcNAcylation and phosphorylation may affect the same or overlapping sites within PER. For example, DBT-mediated degradation of PER is delayed by elevating OGT levels in cultured *Drosophila* cells. OGT-mediated glycosylation may similarly delay PER phosphorylations by GSK-3 and CK2 that promote nuclear accumulation as described above. Consistent with this view, increased levels of PER glycosylation resulting from OGT overexpression delay the timing of PER nuclear accumulation and are associated with long-period circadian rhythms in adult *Drosophila*. Knockdown of OGT in adult flies produces short-period circadian rhythms that are accompanied by an advanced phase of PER nuclear accumulation (41).

OGT conditional knockout mice have short-period circadian behavioral rhythms (42). Thus, as in *Drosophila*, OGT activity appears to play an important role in setting the period length of the mammalian circadian clock. In mice, GSK-3β phosphorylates, and thereby activates, OGT, with a circadian rhythm (42). GSK-3β itself is phosphorylated on Ser9 with a robust circadian rhythm. Ser9 phosphorylation inhibits GSK-3β activity, which has been observed to cycle, as determined by its circadian phosphorylation of a great variety of substrates in several mouse tissues (42). One response to this regulation is rhythmic PER2 glycosylation in the mouse. In particular, *O*-GlcNAcylation of PER2 S662, S665, and S668 blocks CK1-dependent PER2 phosphorylation at these sites (42). The inability to phosphorylate human PER2 S662 has been linked to altered function of PER2 as a transcriptional repressor in a form of familial advanced sleep phase syndrome (reviewed in Reference 22).

In cultured mouse fibroblasts, levels of PER2 *O*-GlcNAcylation, and reciprocally phosphorylation, are altered in response to changing concentrations of glucose in the media; high levels of glucose promote

OGT-dependent PER2 *O*-GlcNAcylation (42). These observations indicate that PER2 *O*-GlcNAcylation may directly function as a metabolic sensor that can adjust the phase of circadian rhythms in response to the varying availability of certain nutrients.

NOVEL PROTEIN PARTNERSHIPS LINK CIRCADIAN CLOCKS TO ENVIRONMENTAL CHANGES AND TO METABOLIC AND DEVELOPMENTAL PATHWAYS

Changing interactions among clock proteins are required to sustain cycles of gene activity. The transcription factors dClock (dCLK) and Cycle (CYC) in flies (CLOCK and BMAL1 in mammals) form a complex that activates expression of the *per* and *tim* genes. Subsequently, physical associations of PER and TIM proteins in *Drosophila* [PER and Cryptochrome (CRY) in mammals] allow accumulation, nuclear localization, and feedback repression (**Figure 1*a*,*b***). PER/TIM and PER/CRY produce negative feedback by direct physical associations with the above activators. In mammals, CLOCK functions as a histone acetyltransferase (HAT). Thus, its ability to activate expression of genes such as *Per* and *Cry* may reflect, at least in part, its local modification of chromatin (43). Nevertheless, the focus of CLOCK's HAT activity is not limited to histones, because CLOCK acetylates its partner, BMAL1. This modification enhances recruitment of CRY so that the HAT activity is involved in both activation and repression of target gene activity (44).

Photoentrainment

Biological clocks quickly adapt to changes in the environment. An especially potent environmental signal is light, which resets the *Drosophila* circadian clock by regulating the abundance of the TIM protein (**Figure 1*a***). Early studies of the *Drosophila* clock revealed advances or delays in the phase of the circadian rhythm in response to short pulses of daylight. The amplitude and direction of the phase shift reflected the time of day when the light pulse was delivered. We now know that these responses depend on an interaction between TIM and CRY. In flies, CRY functions as an intracellular photoreceptor related to the DNA photolyases (PLs; reviewed in more detail in the section below titled Cryptochromes and in Reference 45). All CRYs carry a flavin adenine dinucleotide (FAD) chromophore. The redox state of FAD is affected by blue light; in *Drosophila*, in the presence of blue light, CRY binds to TIM. This association recruits JETLAG (JET), an E3 ubiquitin ligase, leading to ubiquitination and rapid degradation of both TIM and CRY (46). Consequently, a pulse of light delivered in the early night delays the phase of the circadian clock by transiently depleting TIM. However, at that time of night TIM can quickly reaccumulate due to high levels of *tim* RNA. In contrast, a light pulse that occurs just prior to dawn advances the clock: TIM is depleted, but *tim* RNA levels are too low at that time of night for new TIM synthesis during the same molecular cycle (27, 28).

Metabolic and Developmental Signaling

In addition to being responsible for programmed environmental responses, several clock proteins directly associate with proteins engaged in metabolic and developmental signaling. One such interaction involves AMP-activated protein kinase (AMPK). This kinase acts as a nutrient sensor by phosphorylating various protein substrates in response to activation by high intracellular concentrations of AMP versus ATP or by increasing levels of calcium, both of which are responses to limited availability of intracellular glucose. In mammals, CRY1 is an AMPK substrate, and phosphorylation by AMPK kinase destabilizes CRY1 (47). PER2 also appears to be a target for circadian regulation by AMPK in mammals; as reviewed elsewhere (48), PER2 is destabilized by CK1ε-directed phosphorylation, and CK1ε is a regulated substrate of AMPK. Both AMPK activity and subcellular localization vary with a circadian rhythm that has been linked to

the circadian expression of mRNA encoding a regulatory subunit of the kinase AMPKβ2 (47). Therefore, nutrient availability influences the phase of the mammalian circadian clock through altered CRY1 and PER2 accumulation, which in turn affects rhythms of AMPK and nutrient sensing.

Sirtuins (SIRTs) and poly(ADP-ribose) polymerase (PARP) convey metabolic signals to the circadian clock in mammals. SIRT1 is a NAD^+-dependent deacetylase that has been connected to the regulation of longevity, genome stability, and transcriptional silencing (49, 50). Schibler and colleagues (51) have found that SIRT1 accumulates with a circadian rhythm and physically associates with PER2 and CLOCK/BMAL1 complexes. PER2 is deacetylated by SIRT1, a modification of PER2 that causes its degradation. Sassone-Corsi and colleagues (52) have provided evidence that SIRT1 also deacetylates BMAL1 and histone targets of CLOCK's HAT activity.

PARP-1 is a NAD^+-dependent ADP ribosyltransferase whose activity cycles with a circadian rhythm. PARP-1 binds to and poly(ADP-ribose)-ylates CLOCK, altering its interaction with PER, CRY, and its DNA targets (53). Given that cellular NAD^+ levels must be coupled to metabolic activity, changing profiles of cellular NAD^+ should affect patterns of PER2 accumulation and loss, as well as BMAL1 activity through SIRT1, while affecting CLOCK activity through PARP-1. Both pathways can be expected to influence the phase of the circadian clock. As in the case of AMPK interactions with CRY1, circadian rhythms of the metabolic sensors SIRT1 and PARP-1 are also influenced by their own activity. Indeed, for SIRT1, this feedback may be observed at yet another level because SIRT1/CLOCK/BMAL1 complexes directly regulate transcription of *nicotinamide phosphoribosyltransferase* (*Nampt*), which encodes a rate-limiting regulator of the NAD^+ salvage pathway (54). Thus, SIRT1 appears to influence the circadian production of its coenzyme.

Nuclear receptors (NRs) are central regulators of reproductive function, development, metabolism, and immune responses. They are deployed in a ligand-dependent fashion as regulators of gene expression. This large collection of ~50 receptors in mammals affects the expression of hundreds to thousands of genes in diverse tissues. More than half of these receptors accumulate with a circadian rhythm (55). Reciprocal regulation of circadian rhythms by NRs is also widespread, and certain NRs directly bind to and regulate expression of circadian clock genes, including *Per1*, *Per2*, and *Bmal1* (56). Cross talk connects AMPK signaling and NR function, given that the function of some NRs is affected by AMPK-directed phosphorylation (56).

Regulation of NR signaling is also influenced by physical interactions with circadian clock proteins (**Figure 3**). For example, the glucocorticoid receptor (GR), which is involved in stress responses, glucose homeostasis, and immune function, can physically associate with CRY1 or with CRY2 in a manner that is stimulated by the presence of glucocorticoids (57). These associations appear to alter receptor function by rhythmically repressing GR activity with respect to a subset of target genes: Cultured cells derived from *Cry*-deficient mice show elevated expression for hundreds of genes, indicating that in wild-type mice CRY interactions with GR substantially limit the number of targets induced by hormones.

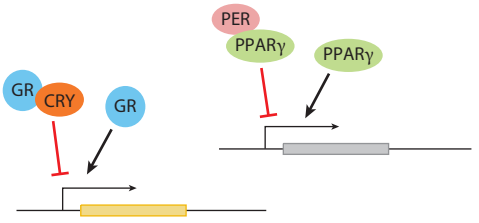

Figure 3

Cryptochrome (CRY) and Period (PER) physically associate with certain nuclear receptors (NRs) and alter NR function. CRY1 and CRY2 interactions with glucocorticoid receptor (GR) lead to circadian rhythms of repression and activation for some GR target genes. PER2 similarly confers oscillating patterns of expression on genes regulated by peroxisome proliferator–activated receptor γ (PPARγ) during adipocyte differentiation.

Because CRY proteins are recovered from promoters of GR-responsive genes following hormone administration, they appear to function as heterologous GR partners that bind to GR response elements on the affected target genes (**Figure 3**) (57). Thus, glucocorticoid signaling is tied to the circadian clock in mammals at multiple levels: Hormone levels themselves fluctuate with a circadian rhythm, receptors accumulate with a rhythm, and receptor function oscillates in response to rhythmically produced CRY1 and CRY2.

Some NRs are also regulated by physical associations with mammalian PER (mPER) proteins (**Figure 3**). For example, the NRs peroxisome proliferator–activated receptor α (PPARα) and REV-ERBα physically interact with PER2 in hepatocytes to regulate *Bmal1* expression (58). PER2 appears to function at one time of day as a repressor of *Per2* expression (through PER2–BMAL1 or PER2–REV-ERBα protein interactions) and at other times within the circadian cycle as an activator of *Per2* expression (through PER2–PPARα interactions). PER2 also regulates PPARγ during adipocyte differentiation (59). This control requires a specific physical interaction with PPARγ, impairs PPARγ recruitment to regulatory elements of PPARγ targets, and represses PPARγ transcriptional activity. In contrast to wild-type mice, *Per2*-deficient mice show a broad activation of adipogenic genes in their white adipose tissue, further indicating a significant role for PER2–PPARγ interactions in normal adipocyte development and function. *Per2* deletion alters both the circadian phase and the amplitude of rhythmically expressed PPARγ targets in white adipose tissue (59). PER3/PPARγ plays a related role in adipocyte differentiation (60).

CORE OSCILLATOR STRUCTURAL CONSIDERATIONS

There are common structural features among the transcriptional activators and repressor proteins that compose the core oscillators of the fly, fungal, and mammalian clocks (**Figure 4**). Plant oscillators are also composed of autoregulatory feedback loops; however, the components therein diverge considerably from those of the other systems, so we direct the reader elsewhere for a discussion about them (61, 62). A hallmark of clock oscillator proteins is the PAS (Per/Arnt/Sim) domain, whose signature sequence was first identified as a dimerization domain in *Drosophila* PER (dPER) (63). PAS domains are widespread modules of many signaling proteins in both eukaryotes and prokaryotes (64–67). They are typically composed of ~120–150 residues that fold into five antiparallel β-strands (**Figure 5*a***). A helical cluster (Eα–Fα) that often forms a cofactor binding pocket packs across one face of the β-sheet, and a more variable helical region formed from C- and/or N-terminal (Ncap) segments associates on the opposing side (**Figure 5*a***). [A note on PAS nomenclature: Most fields alphabetically designate the secondary structural elements regardless of type, e.g., Aβ-Bβ-Cα-Dα-Eα-Fα-Gβ-Hβ-Iβ; others, as is common with PER, separate the α-helices and β-strands into unique naming categories (**Figure 5*a***).] Circadian clock proteins, such as CLK, CYC, PER, CLOCK, BMAL1, NPAS2, mPER1–3, WC-1, and WC-2, all contain PAS repeats, where a PAS-A domain and a PAS-B domain are tightly coupled in sequence and association (**Figure 4**). In all cases, the PAS-AB repeats compose only an ~300-residue segment of a much larger protein (>1,000 residues). The remaining polypeptide is composed of various DNA-binding motifs, chromatin modification domains, phosphorylation/glycosylation centers, coiled coils, poorly ordered regions, and stretches of low sequence complexity (**Figure 4**). Indeed, the repetitive stretches of PER are one of the first documented cases of intracellular protein sequences that did not match expectations for a globular structure (68, 69). The other major oscillator components of the clock system, TIM (*Drosophila*), FRQ/FRH (*Neurospora*), and CRY (*Drosophila*, mammals), belong to classes of entirely different proteins that do

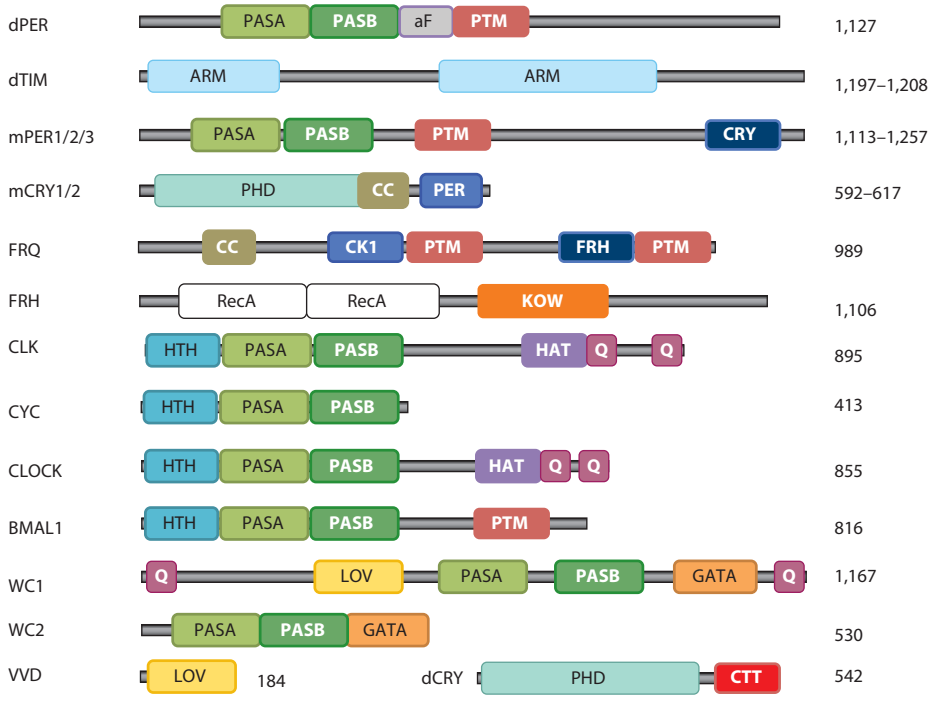

Figure 4

Domain architectures of core circadian clock proteins. The major structural domains are as follows: PASA and PASB, Per/Arnt/Sim (PAS)-AB repeat; PTM, posttranslational modification by phosphorylation, glycosylation, and/or acetylation; ARM, armadillo helical repeats; CRY, Cryptochrome-binding; PHD, photolyase homology domain; CC, coiled-coil; PER, Period-binding; CK1, casein kinase 1–binding; FRH, FRH-binding; RecA, ATPase; KOW, RNA-binding; HTH, helix-turn-helix DNA-binding domain; HAT, histone acetyltransferase; Q, Gln-rich transcriptional activation; LOV, light-oxygen-voltage-sensing domain; GATA, zinc-finger DNA-binding; CTT, C-terminal tail. The protein size in residues is shown on the right. Other abbreviations: d, *Drosophila*; m, mammalian; TIM, Timeless; VVD, Vivid; WC, White-Collar.

not contain PAS domains, as discussed in the following sections.

PAS Domains

Crystal structures have been determined for the isolated PAS-AB repeats of PER (70, 71), mPER1 (72), mPER2 (73), mPER3 (72), and the CLOCK/BMAL1 heterodimer (**Figure 5*b***) (74). All these proteins are dimeric, and although the domains themselves adhere to the standard PAS architecture, they have surprisingly different arrangements within the dimers. PER forms a head-to-tail dimer in which PAS-A interacts with PAS-B′ of the opposing subunit (70, 71). A highly conserved Trp residue in the loop connecting βD to βE of PAS-B packs near the entrance of the αA–αC pocket that often binds cofactors (**Figure 5*b***) (70). A PAS-B C-terminal helix, termed αF, wraps back around to latch against the β-sheet of PAS-A. The interaction between αF and PAS-A appears highly dynamic because it can associate with varied helical registers (71), and some structures show it to be completely released from the PAS-A domain (**Figure 5*b***) (70). The αF-helix directly contacts the site of the per^L mutation (V234D), which causes a lengthened clock period (75). Residue substitution on αF (M560D) disrupts PER homodimerization both in vitro (73) and in flies (76). M560D slightly lengthens the period and retards nuclear

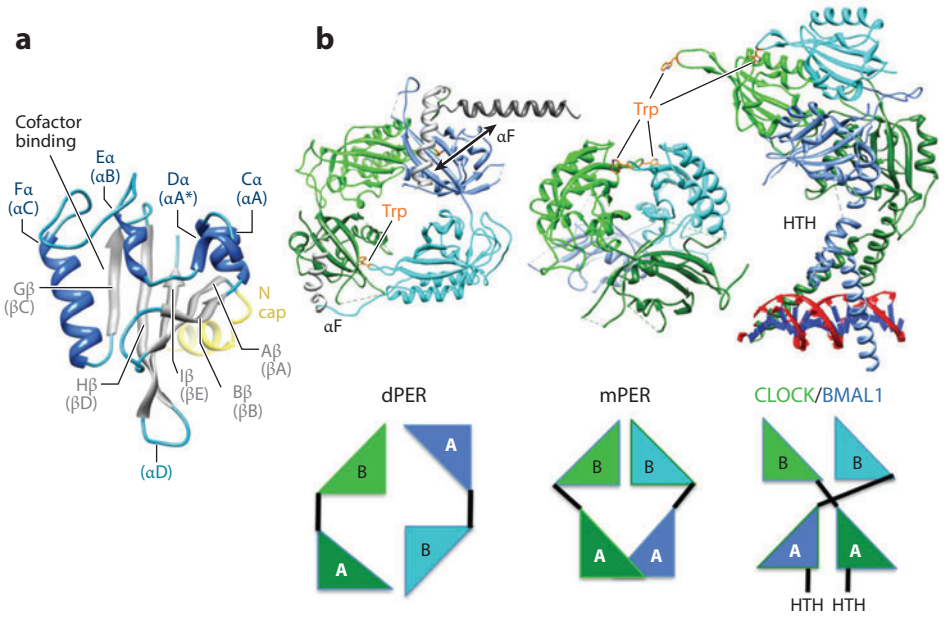

Figure 5

Structures of Per/Arnt/Sim (PAS)-AB repeats in clock proteins. (*a*) Archetypal PAS structure with elements labeled by standard nomenclature and that used with Period (PER) proteins (in parentheses). (*b*) (*Top*) Variation in PAS-AB organization displayed by *Drosophila* PER (dPER), mammalian PER (mPER), and CLOCK/BMAL1. (*Bottom*) Corresponding schematics. For dPER, the αF-helix wraps the PAS-AB repeat but shows bound and released conformations in different structures. The structures of mPER1 and mPER3 (not shown) are very similar to that of mPER2. The model of CLOCK/BMAL1 combines the structures of helix-turn-helix (HTH)/PAS-AB and the HTH/DNA complex (DNA is in red). The conserved Trp residues (*orange*) on the Hβ–Iβ loop have very different environments in dPER, mPER, and CLOCK/BMAL1. Protein Data Bank identifiers: dPER, 1WA9 and 3RTY; mPER2, 3GDI; CLOCK/BMAL1, 4F3L and 4H1O.

accumulation of PER (76) but does not appear to affect interactions with TIM, which instead localize along the β-sheet of PAS-A (73, 75). Although M560D does not affect TIM interactions, the per^L substitution does (75); this finding is curious because both changes perturb the same homodimer interface. Thus, nuclear entry of PER, and therefore repression of CLK/CYC, may depend on both PER homodimerization and TIM binding, which are coupled in a manner that is not yet understood at the molecular level.

Unlike the head-to-tail arrangement of dPER, the PAS repeats of all three mPER proteins show a much different association mode (**Figure 5*b***), wherein the PAS-B β-sheets stack in an antiparallel fashion mediated by the Trp in loop βD–βE conserved with PER (72, 73). In the mPERs, the PAS-A domains curl around in opposite directions from PAS-B to form a compact structure; the juxtaposition of PAS-A to PAS-B is more expanded in mPER1 and mPER3 compared with that in mPER2. Mutation of the conserved Trp again affects mPER dimerization both in vitro and in vivo (72). An interaction motif for NRs, LXXLL (58), is found on the βE-strand of PAS-A in all mPERs, although the Ncap occludes this region to various extents in the respective structures. This position on the backside of the PAS-A β-sheet is similar to that of the TIM-interacting residues of PER PAS-A (73).

The structure of the mammalian CLOCK/BMAL1 heterodimer displays yet another

arrangement of the PAS-AB repeat (**Figure 5*b***) (74). In this case, PAS-AB associate in a head-to-head parallel contact, dictated by the necessity of the N-terminal helix-turn-helix (HTH) DNA-binding motifs recognizing the palindromic sequence of the E-box (77, 78). The PAS-A domains form a heterodimer that is similar in structure to bacterial PAS dimers such as NifL (79). Surprisingly, the PAS-B domains are not related by similar twofold symmetry; instead, they align in a back-to-front association. This alignment engages the conserved Trp of BMAL1 with the αB–αC pocket of CLOCK (as in PER), but because CLOCK PAS-B is rotated by 180°, the CLOCK Trp is exposed and perhaps made accessible for interaction with the mPER/mCRY (mammalian CRY) repressor complex (74). A structure of the HTH heterodimer of CLOCK/BMAL1 bound to DNA reveals the expected recognition of the palindromic E-box (**Figure 5*b***). Each N-terminal helix inserts into the major groove and primarily contacts 3 bp (80). However, the structure also reveals that bases +1 and −1 of the palindrome are recognized by the protein. This breaking of symmetry explains the ability of the heterodimer to distinguish orientation and to recognize noncanonical E-box sequences such as those controlling *mper2* (77).

On the basis of sequence, the *Drosophila* dCLK/CYC activation complex is expected to be quite similar to that of CLOCK/BMAL1. However, the structure of the related White-Collar Complex [WCC (81–83)] of the fungal clock is more difficult to predict. Both WC-1 and WC-2, the two transcription factors that compose the WCC, also contain a PAS-AB repeat (81, 84), but in this case the PAS repeats are N-terminal to GATA-type zinc-finger DNA-binding domains (85–87). WC-1 also contains an additional PAS domain of the light-oxygen-voltage (LOV) subtype that binds a flavin nucleotide and is responsible for light-induced gene activation by the WCC (84, 88–90). In general, the sequences of the WC-1 and WC-2 PAS domains diverge substantially from those of CLOCK or BMAL1 and in fact are closer to those of PER. However, the head-to-tail PER association would not allow each subunit of the WCC to recognize a short segment of DNA as do the head-to-head arrangements of CLOCK/BMAL1 and CLK/CYC. Indeed, the light-responsive elements (LREs) of the fungal clock contain imperfect palindromes of the GATA recognition motif that can occur in direct repeats separated by ~17 nt (85, 89). Thus, despite a similar domain construction, the architecture of the WCC may not parallel that of the animal transcriptional activators. By analogy to PER/NR associations, WC-1 interacts with a histone acetyl transferase via LXXLL motifs that localize to the PAS-AB repeat (91).

Cofactor Binding to PAS

Many PAS domains bind cofactors or substrates to mediate the sensing of ligands, light, or redox (64–67). Cofactor binding by PAS-containing clock proteins has also attracted attention. NPAS2, a paralog of CLOCK, was identified as a gas-sensing protein (CO and NO) that depends on heme binding for activity (92). Since this discovery, several studies have characterized the in vitro heme-binding properties of the PAS domains from NPAS2, mPER1–3, and CLOCK (72, 93–100). However, an examination of heme binding by mPER2 found that the interaction is largely nonspecific, with properties characteristic of proteins that are not known to have functional interactions with heme (97). In vivo effects of heme on the clock are complicated by the involvement of the non-PAS NRs REV-ERBα and REV-ERBβ, which bind heme as their ligands and regulate the expression of *Clock*, *Bmal1*, and *Npas2* (101–105). Furthermore, the structures of CLOCK and mPER1–3 would have to undergo substantial changes in conformation and oligomeric state to accommodate heme in the PAS cofactor pocket. A novel heme-binding motif may also appear in the C-terminal domain of the mPERs (106). Mutants therein affect the association of exogenously added hemin and mPER stability. Recently, investigators used a fluorescent dye that is sensitive to by-products of heme-based oxidations to detect the presence of heme

in nuclear-localized NPAS2, but not CLOCK (107). In summary, the mammalian clock receives input from heme through the REV-ERB proteins; whether additional heme signals are integrated through the core oscillator proteins is an open question.

Structure Peripheral to PAS

Outside their PAS domains, dPER and mPER contain dissimilar regions with characteristics of largely unstructured sequences and modification centers, with the exception of an ~30-residue conserved segment that directs phosphorylation by DBT and the casein kinases (108, 109). Immediately C-terminal to the PAS-AB repeat in dPER are the so-called per-short domain and the per-short downstream domain, which contain several phosphorylation sites, one of which is the position of the *perS* mutation [Ser589 (10, 110, 111)]. Missense mutations here (and even deletions limited to ~20 amino acids encompassing this region) dramatically shorten the period length of the fly clock. A hierarchical sequence of phosphorylation in these domains may gate PER degradation by controlling the conformational state of the protein. A more open phosphorylated state may favor access to DBT and the SLIMB ubiquitin ligase, the latter of which recognizes phosphorylation sites at the N terminus (7, 8). The mCRY interaction region localizes to the C termini of the mPERs, where it is presumed to assume a helical domain (112).

In CLOCK, the HAT domain C-terminal to the PAS-AB repeat has some sequence similarity to a conserved motif in a structurally defined histone acetyl transferase [yeast ESA1 (43)]. Several residues involved in binding acetyl-CoA are conserved between CLOCK and ESA, and mutations therein affect HAT activity of CLOCK (43). However, outside of this active site region there is little homology between the two proteins, and the entire ESA1 acetyltransferase domain could not be accommodated by the CLOCK sequence (113). Thus, the overall architecture of the CLOCK HAT domain could be quite different than those of known acetyltransferases.

Helical Repeats in TIM

TIM is also a large protein that is predicted to have low order across its length. With some differing interpretations (114–118), bioinformatic analyses have identified TIM as a helical-repeat protein that may form two major domains of armadillo (ARM) repeats (118). In this scenario, each ARM repeat comprises three helices in a roughly triangular arrangement, and subsequent repeats layer on top of each other in a so-called α-helical solenoid (114). The ARM domains are separated by a low-complexity region, which also contains repetitive elements. C-terminal to the second ARM domain is another low-complexity region followed by three two-helix structural motifs similar to those found in prenyltransferases (118). TIM residues 447–914, which localize within the second predicted ARM domain, are important for binding PER (75). Given the tendency of the PER PAS domains to associate helices across their respective β-sheets, a TIM ARM helix may associate with PER PAS-A in a similar fashion.

The Many Faces of FRQ

Other than PER, the best-studied clock repressor protein is probably Frequency (FRQ), a key oscillator component of the fungal clock (for reviews, see References 119–121). FRQ plays the role of PER/TIM or mPER/mCRY in *Neurospora crassa* and is associated with the frequency-interacting RNA helicase (FRH). FRQ has two major structured domains: One binds FRH, the other casein kinase 1a (CK1a, analogous to DBT). FRQ also contains a coiled-coil domain for dimerization that is essential for clock function (122). In addition, two PEST degradation sequences are centers for phosphorylation and act to coordinate proteasome-mediated degradation of FRQ (123, 124). FRQ mRNA undergoes alternative splicing to produce long and short versions of the protein (lFRQ and sFRQ) that differ by 100 residues at the N terminus (125). An increase in temperature produces more lFRQ and is correlated with temperature compensation of the clock

(126–128). FRQ has been referred to as a phosphorylation platform because it contains a vast number of sites (>100) whose phosphorylation states vary over the circadian day and correlate with activity and degradation (129). Interestingly, the pattern of phosphorylation is spatially and temporally heterogeneous; the middle of the protein is rapidly phosphorylated after synthesis, followed by the PEST sequences toward the end of the day and then the N terminus of the lFRQ toward the end of the circadian cycle (129). Rather than single sites having specific functions, a cumulative effect of phosphorylation appears to drive conformational changes in the molecule that control associations of the ordered N- and C-terminal domains and possibly other factors (130). Two short stretches of amphipathic helices (residues 319–326 and 488–495) were identified as key elements of the FRQ–CK1a interaction domain (FCD1 and FCD2). Both are required for binding CK1a and inducing phosphorylation (130). Unphosphorylated FRQ adopts a closed conformation held by electrostatic interactions between the basic N-terminal domain (residues 1–355) and the acidic remainder. Progressive phosphorylation of N-terminal regions by CK1a negates this attraction to promote an open conformation that reveals the central PEST1 sequence for degradation.

The long unstructured regions of FRQ have special properties that place unexpected constraints on their production. These segments are encoded by relatively rare codons, which slow their rate of synthesis (131). If codon usage is switched to favor abundant tRNAs, FRQ levels are increased, phosphorylation patterns change, and circadian rhythmicity is lost in constant darkness. Codon optimizations at the N terminus of FRQ alter stability and phosphorylation in ways that differ from optimizations at the middle of the protein. Nevertheless, codon optimizations in both locations increase overall FRQ protein levels. Improper codon usage may affect the rate of translation for segments that rely on the influence of other factors to control folding and the modification state (131).

FRH, the Partner of FRQ

FRH belongs to the Dob1p/Mtr4 family of RNA helicases that function in RNA surveillance as part of the TRAMP complex (132–134); the yeast versions have been crystallographically characterized (135, 136). Mtr4 has an ATPase core that is typical of other DExH helicases but also contains a KOW domain—as found in ribosomal proteins that bind 23S ribosomal RNA. FRQ always associates with FRH, which binds to FRQ at a specific interaction domain toward the C terminus of the protein. FRH stabilizes FRQ but also interacts with *frq* mRNA and influences the stability and length of its poly-A tail (137). FRH interacts with the *Neurospora* homologs of the TRAMP complex and with exosome components (137). FRH is essential for RNA metabolism, but there are viable mutants that leave these properties intact while still causing clock dysfunction (134).

MECHANISMS OF LIGHT SENSING

LOV Domain Proteins

In the fungal clock, light induces *frq* transcription in a fashion that depends on the WCC (reviewed in References 124, 138, and 139). The components of the WCC, WC-1 and WC-2, associate and bind at LREs in the regulatory regions of *frq* and other clock-control genes (89, 90, 140, 141). WC-1 contains a blue light–sensitive, LOV-sensing domain of the PAS superfamily that binds a flavin cofactor (84, 85, 89, 90). The WCC binds to LREs in both the presence and absence of light, but the size of the complex increases upon light exposure (89, 140) due to oligomerization of the LOV domains (142).

The WC-1 LOV domain is very similar in sequence (80% sequence similarity over 150 residues) to a second clock-associated light sensor in *N. crassa*, Vivid (VVD) (143). VVD opposes the action of the WCC to allow light adaptation (143–145). The WCC induces *vvd* expression, and VVD subsequently inhibits the

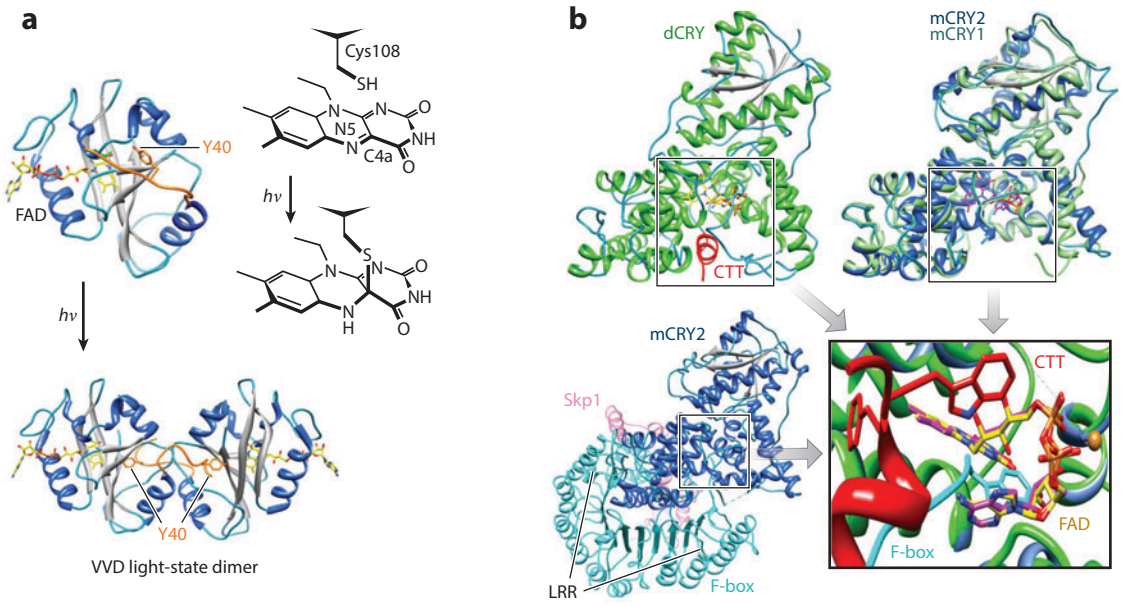

Figure 6

Flavin-binding clock proteins. (*a*) The fungal Vivid (VVD) light sensor undergoes light-induced dimerization. In the light-activated dimer (*bottom*), the N-terminal latch (*orange*) swings out to engage Y40 with the adjacent subunit. This structural change is driven by a cysteinyl-flavin adduct formation. (*b*) The Cryptochrome (CRY) flavin pocket is a hot spot for interactions. (*Top left*) The C-terminal tail (CTT) (*red*) binds adjacent to the flavin in *Drosophila* CRY (dCRY). (*Bottom left*) The C terminus of the F-box protein Fbxl3 (*cyan*) inserts into the empty flavin pocket of mammalian CRY2 (mCRY2), and the leucine-rich repeats (LRRs) surround the last helix of the photolyase homology domain. (*Top right*) Flavin binds to mCRY2 in a similar mode as dCRY; *apo*-mCRY1 and mCRY2 have similar structures. (*Bottom right*) Close-up of a flavin pocket showing superposition and juxtapositions of dCRY flavin adenine dinucleotide (FAD) (*yellow*); dCRY FFW motif of the CTT (*red*); mCRY2 FAD (*magenta*); and F-box C-terminal Trp residue (*cyan*). Protein Data Bank identifiers: VVD monomer, 2PD7; VVD dimer, 3RH8; dCRY, 4GU5; mCRY2, 4I6G; mCRY1, 4K0R; Skp1/Fbxl3/mCRY2, 4I6J.

WCC by binding directly to it in a light-dependent fashion (139, 142). VVD attenuates WCC function to a degree that depends on the level of light exposure (139). Consequently, the *Neurospora* clock receives a pulse of active WCC at dawn that is quickly attenuated. Domain-replacement studies demonstrate that the core PAS domain of VVD can functionally substitute for that of WC-1 (140). LOV domain proteins are broadly represented in photosensory roles throughout biology, and their structures and photocycles are well characterized (146–149). Although many features are conserved among all LOV domain proteins, the mechanisms by which downstream effectors are stimulated differ depending on the particular class of LOV proteins. In general, absorption of blue light by the associated flavin (which can be FAD or FMN) produces a covalent adduct between a conserved cysteine residue on Eα and the C4a position of the isoalloxazine ring of the flavin (**Figure 6*a***) (146–149). Structures of VVD in the dark-adapted, light-adapted adduct, as well as signaling states (150–153), show that formation of the adduct propagates to larger structural rearrangements that culminate in protein dimerization (**Figure 6*a***) (151, 154, 155). VVD has a typical LOV/PAS structure; FAD is bound in the PAS cofactor pocket between the β-sheet, Eα, and Fα. The Ncap of VVD is composed primarily of a β-strand, an α-helix, and an N-terminal latch that reaches back around the β-sheet to the adenosine moiety of FAD. As shown in structures of illuminated crystals

grown from dark-state VVD, formation of the Cys adduct effectively reduces the flavin ring and results in protonation of flavin N5. Flavin protonation alters the conformation of conserved Gln182, whose perturbed interactions propagate to the β-strand and hinge leading to the Ncap. The net result is a modest shift of the α-helix and β Ncap elements. Nonetheless, the crystalline environment prevents any larger-scale changes to the structure. An investigation of factors affecting the VVD photocycle led to the recovery of variants that stabilize the flavin adduct state and alter the lifetime of the light-adapted dimer (150). Subsequent crystallization of these species revealed subunit changes similar to those observed on illumination of dark-state crystals, but in addition, the N-terminal latch completely releases and reaches across to engage another VVD subunit (**Figure 6*a***) (153). A conserved Tyr residue in the N-terminal latch inserts into the same hinge between the β-strand and the Ncap that promulgates latch release. Mutation of this Tyr and other interface residues in the dimer structure abrogates or diminishes the biological activity of VVD in vitro and in vivo (153). Molecular dynamics simulations of the VVD adduct state on the nanosecond timescale have suggested a different activation mechanism that involves similar regions of the protein but also much larger conformational changes of Gln182 than have been observed in crystal structures (156).

Notably, most of the structural features that mediate the VVD conformational response are conserved in the WC-1 sequence. Thus, a similar light-induced dimerization mechanism is very likely to be operative and responsible for recruitment of additional WC-1 subunits to the transcriptional activation complex (153). Indeed, the LOV domains of WC-1 also undergo light-dependent dimerization and VVD interferes with this process (142, 153). These findings gave rise to the idea that VVD may exert its inhibitory effect by producing a heterodimer with the WC-1 LOV domain that resembles the VVD light-state dimer (139, 142, 151, 153, 157). Such an interaction would break up the higher-order WC-1 complex formed with light and diminish WC-1 transcriptional activation. Attractive as such a detailed mechanism for light adaptation is, a VVD/WC-1 heterodimer remains to be structurally characterized.

Cryptochromes

CRYs are flavin-binding proteins that closely resemble PL DNA repair enzymes (158–160). CRYs play quite different roles in the circadian clocks of mammals, insects, and plants (158–160). *Drosophila* CRY (dCRY) is the light-entrainment sensor of the fly clock, although it also has non-clock-related functions that include magnetosensitivity (161–163). Mammals contain two CRY proteins (mCRY1 and mCRY2) that instead interact with mPERs as core elements of the circadian clock and do not mediate light responses (**Figure 1*b***) (160, 164–167). PLs repair cross-linked pyrimidine dimers formed in DNA by UV radiation. PLs rely on two cofactors, FAD and a second antenna cofactor that is usually a pteridine moiety [methenyl-trihydrofolate (MTHF)] or a deazaflavin [hydroxy-ribo-deazaisoalloxazine (HDF)]. In PLs, light energy absorbed by the antenna cofactor is relayed to reduced FAD by energy transfer. The excited FAD then injects an electron into the DNA lesion and breaks the pyrimidine linkage by a cyclic electron-transfer reaction (158, 168). Although at least some CRYs bind FAD similar to PLs, the PL DNA repair mechanism is not thought to participate in CRY functions within the clock (158–160, 167).

The CRY/PL family can be grouped into several subfamilies on the basis of sequence and function: (*a*) cyclobutane pyrimidine dimer (CPD) PLs (classes I and II); (*b*) 6-4 PLs; (*c*) CRY-DASH proteins, which are single-stranded DNA PLs; (*d*) plant CRYs; and (*e*) animal CRYs (158, 169–171). All family members are composed of a common structural fold consisting of an N-terminal α/β nucleotide–binding domain connected to a C-terminal α-helical domain through a flexible linker. Animal CRYs can be further subdivided by sequence and function into type I, as found

in *Drosophila*, and type II, as found in mammals (172–176) and other insects (174). Different CRYs are distinguished by C-terminal extensions (CCEs) that vary considerably in sequence and length (158, 169–171).

As discussed above, dCRY regulates circadian function by targeting TIM for ubiquitin-mediated degradation in the presence of light through action of the E3 ubiquitin ligase JET (177–179). Light also causes dCRY degradation (177–180) through action of BRWD3 E3 ubiquitin ligase (181). dCRY engages TIM in the presence of light. Binding is necessary but not sufficient to degrade TIM (180, 182). The light-dependent recognition of TIM by dCRY involves changes at the unique CCE of dCRY (180). For example, dCRY with the 19 C-terminal residues removed (CRY_M) binds TIM both in the dark and in the light and greatly reduces clock entrainment under normal levels of light (180). In vertebrates, CRY enters the nucleus and interacts directly with CLOCK/BMAL1 (164, 183–185). The mCRYs are targets of the E3 ubiquitin ligase complex SCF^{Fbxl3}; the F-box protein Fbxl3 directly recognizes and marks CRY for degradation (186–188). AMPK phosphorylates mCRY1 to promote binding of Fbxl3 (47), whereas a homologous F-box protein can compete with Fbxl3 to prevent degradation and effectively lengthen clock period (188).

The structure of full-length dCRY reveals a typical PL-type fold in which the α/β domain is devoid of cofactor and the α-helical domain binds FAD in a deep pocket (**Figure 6*b***) (189–191). Although there is some evidence that dCRY binds MTHF (192), key residues involved in this interaction are not conserved with PLs, and the structure of the antenna region differs considerably in CRYs compared with PLs. Currently, there is no known biological function for a CRY antenna cofactor, and no known structures contain antenna cofactors (189, 192, 193).

C-terminal to the photolyase homology domain (PHD), a small loop connects to the so-called C-terminal tail of dCRY (CTT) (residues 528–539). The CTT forms a helix containing a conserved FFW motif that occupies a groove analogous to that where damaged DNA substrates bind in PLs (189–191). The sequence register of the CTT with respect to the PL-homologous groove in the first reported dCRY structure (189) was subsequently revised to yield an improved model (191). This update recognized a shift by two residues of the CTT sequence register within the PL-homologous groove (191). The revised structure was confirmed by an independent study of a dCRY variant that was slightly altered in the phosphate loop that helps sequester the flavin pocket (190). Nonetheless, in all cases the CTT is positioned to respond to light-induced redox changes at the FAD and appears to fulfill the role of a classic autoinhibitory motif (**Figure 6*b***). Indeed, proteolytic protection assays indicate that the CTT becomes more exposed upon light exposure (190, 194).

The PHD structures of mCRY1 and mCRY2 closely resemble each other and are also quite similar to that of dCRY (**Figure 6*b***) (190, 195). Differences include the phosphate-binding loops and CCEs, which are largely absent in the current mCRY structures. mCRYs mostly purify without FAD (160), although there are exceptions (196). Nonetheless, FAD binds to mCRY2 after purification, and the flavin-binding mode is analogous to that observed in dCRY (195). In the structure of the ternary complex of mCRY2 with its E3 ubiquitin ligase Flxl3/Skp1, the highly conserved C-terminal projection of Fbxl3 inserts into what would be the flavin binding pocket of mCRY2 (**Figure 6*b***) (195). The F-box protein then wraps 12 leucine-rich repeats (LRRs) around the α-helical domain of mCRY2 to completely surround the C-terminal helix of the PHD. The flavin binding pocket of mCRY has also been revealed as the target for a set of synthetic carbazole compounds that compete with the E3 ligase and thereby inhibit mCRY degradation (197). These interactions were found during a small-molecule library screen for altered expression of a *Bmal1* reporter construct in human osteosarcoma cells. The crystallographic structure of mCRY2 in

complex with such a compound shows how it mimics interactions of both FAD and the C terminus of Fbxl3 with the mCRY pocket (198).

Thus, E3-ligases, autoinhibitory motifs, cofactors (e.g., FAD), and small molecules all compete for the analogous "receptor" pocket in the α-helical domains of CRYs (**Figure 6*b***). The competition among these interactions, which is modulated by flavin redox state (itself influenced by light), is probably the core property underlying CRY functionality. Nonetheless, there is still much to be learned about CRY interactions and the biological role of flavin. In mCRY, the C-terminal helix of the α-helical domain bound by the LRRs of the F-box has also been predicted to form part of a coiled-coil domain. This presumed coiled coil, when contained in an isolated CCE, interacts with C-terminal fragments of BMAL1 (185). mCRY binds to multiple locations on BMAL1 (183–185, 190, 199) and with PAS domains of CLOCK (199). Indeed, the conserved Trp residue on PAS-B of CLOCK that has turned out to the solvent (see the previous section) may be recognized by mCRY (74). Yeast-2 hybrid studies of zebrafish CRY1 (zCRY) indicate that interactions with CLOCK and BMAL1 prevent their ability to dimerize (199). Another study found that zCRY binds to intact CLOCK/BMAL1 on DNA through its PHD but requires reductants for this activity (183, 184). The CCEs of mCRY also mediate interactions with the C terminus of mPER (200).

Regarding the mechanism of light sensing, the chemical states of dCRY that participate in the photoswitch remain unclear; both the anionic semiquinone and a further photoexcited state have been suggested to be the forms that elicit downstream signals (192, 194, 201–204). Photoreduction or chemical reduction to the anionic semiquinone correlates with conformational changes at the CTT that allow for binding to regions of TIM (203); however, dCRY will still promote TIM degradation when an internal Trp residue important for flavin photoreduction in vitro is removed (204). In the case of mCRY, as yet there is no strong evidence for flavin function in vivo, although structural and biochemical evidence is consistent with flavin binding (167, 195, 196). Remarkably, a CPD PL from *Potorous* can rescue clock function in a mouse mCRY1/2 knockout strain (205). The CPD PL contains no CCE, so the requirement for these regions may stem from their ability to influence properties of the PHD, which must itself have properties that are capable of supporting the clock. Perhaps we have come full circle in generalizing the in vivo activities of CRY and PL.

Melanopsin

Although investigators initially thought that CRY proteins might be responsible for light entrainment of the mammalian clock (167), research over the past decade has led to the realization that the principal light sensors are opsins that localize to specialized neurons that lie below the rods and cones of the eye (for reviews, see References 206–210). These cells, termed intrinsically photosensitive retinal ganglion cells (ipRGCs), project directly into the suprachiasmatic nucleus of the hypothalamus and, on their own, provide information about environmental light to this key coordinator of circadian rhythmicity within the brain (211–213). Although ipRGCs function independently of retinal rods and cones, they receive input from the major light-sensing cells. Thus, various light sensors can convey complex photoentrainment signals to the mammalian pacemaker. Photosensing by ipRGCs is distinct from spatial vision and has appropriate properties for monitoring background light-intensity levels, such as high thresholds for light responses, slow temporal responses, a large dynamic range, and enhanced sensitivity to blue wavelengths (212).

Photosensitivity of ipRGCs derives from a unique opsin molecule, melanopsin, that has higher sequence identity with opsins from cephalopods and arthropods (39%) than with vertebrate ocular rhodopsins (27%). Melanopsin was originally identified in the dermal melanophores of photosensitive frog skin (214); orthologs in mouse and human genomes

were then found to localize to ipRGCs by in situ hybridization techniques (212, 213, 215). The circadian clocks of melanopsin knockout mice have altered phase-resetting responses to light pulses (165, 216, 217), whereas mice also devoid of rods and cones show no response to light at all (165, 166). There are two types of melanopsins: *Opn4x*, which is found in all vertebrates except mammals, and *Opn4m*, which is found in mammals (218). Like other opsins, melanopsin belongs to the class of seven-transmembrane α-helical proteins known as G protein–coupled receptors (GPCRs). Opsins bind a retinal cofactor through a Schiff base linkage to a conserved Lys residue (219). Photon absorption causes an isomerization of the chromophore from an 11-*cis* configuration to an all-*trans* configuration. Melanopsins differ from the rhodopsins of rods and cones in several important ways (207, 209, 210): Their action spectra is blueshifted [440–480 nm versus 510–560 nm (209, 220)], they are bleach resistant due to photoregeneration of the 11-*cis* configuration, and they couple to different downstream G protein targets ($G_{q/11}$) than do vertebrate rhodopsins ($G_{i/o}$, transducin).

Sequence comparisons with bovine rhodopsin led to early structural predictions for melanopsin (221). Although the overall sequence identity among divergent opsins is not large (~27%), a 35-residue noncontiguous motif termed the ligand pocket vector (LPV) captures many of the distinguishing characteristics of the classes (222). Conservation of the LPV is high within classes of vertebrate rhodopsins (94–100% identical for rod rhodopsins, 89–100% for *Opn4x*, and 94–100% for *Opn4m*) and correlates well with differences in functionality. Most opsins bind the retinal cofactor among the seven-transmembrane helices in the center of the protein, closer to the extracellular side of the membrane (219). Transmembrane helix 3 and extracellular loop 2 (between helices 4 and 5) sandwich the cofactor. Nonetheless, distinguishing features of melanopsin include alternation of the Schiff base counterion from a Glu to a Tyr (contained on helix 3); charge compensation for the Schiff base by another Glu in extracellular loop 2; an insertion in the third cytoplasmic loop, lengthening of the N and C termini; and alteration of various residues in helices 1–4, increasing the hydrophobic nature of the retinal binding site (221).

More recently, insights into the structure of melanopsin have come from structural studies of a squid rhodopsin [33% identical with melanopsin (223)], in which several distinguishing features of melanopsin are conserved, particularly in the chromophore binding pocket. Like melanopsin, the Schiff base environment of squid rhodopsin contains a Tyr hydroxyl group and an amide side chain supplied by a helix-2 residue. Also like melanopsin, the Schiff base counterion is supplied by a Glu residue on extracellular loop 2. The Glu carboxylate interacts with the chromophore indirectly through the aforementioned amide side chain. The increased hydrophobicity of the retinal binding pocket relaxes the retinal polyene-chain conformation and allows for tighter contact of the chromophore with helix 3 than in vertebrate rhodopsin. Such changes may contribute to the stability of the retinal all-*trans* form. Computational modeling studies of melanopsin based on the squid rhodopsin structure (220) indicate that the Glu counterion forms a hydrogen bonding network with the helix-3 Tyr, the helix-2 amide (Gln), and two water molecules in the vicinity of the Schiff base. This model also suggests that removal of bulky side chains in helices 3 and 7 allows for a more planar chromophore than even that found in squid rhodopsin. Finally, a key substitution of the helix-2 amide from an Asn to a Gln in melanopsin may alter hydrogen bonding around the Schiff base to reduce polarization of the cofactor. These hydrogen bonding perturbations combine with steric effects on the retinal to contract the polyene double bonds and ultimately blueshift the absorbance maximum of the chromophore.

For bovine rhodopsin, structures of intermediate states have helped reveal the mechanism of activation (219, 224). In the structure of metarhodopsin II, isomerization of retinal to the all-*trans* form tilts the β-ionone group of

the chromophore toward helices 5 and 6 (225–227). This tilting bends helix 6 at a conserved Pro residue, extends helix 5, and opens a pocket between these helices and the protein core on the cytoplasmic face. This opened pocket accommodates a peptide derived from transducin in which the C-terminal end of helix 5 binds surrounded by six of seven helices (227). The structure of the β_2-adrenergic receptor in complex with an intact heterotrimeric G protein reveals a similar interaction between helix α5 of the G protein and a cytoplasmic pocket created in part by helices 5 and 6 of the GPCR (228). In the β_2-adrenergic receptor, recognition of helix α5 by the GPCR dramatically alters the conformation of the $G_s\alpha$-subunit, whose two domains crack open to expose the nucleotide binding pocket and facilitate exchange of GDP (228, 229). Studies of the initial stages of squid rhodopsin activation indicate that an early all-*trans* intermediate has a structure similar to that of vertebrate rhodopsin (230). Nonetheless, the later, larger-scale conformational changes necessary for G protein activation remain to be resolved for an invertebrate rhodopsin and certainly for the related melanopsin.

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Errata

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