



Review

How is the inner circadian clock controlled by interactive clock proteins? Structural analysis of clock proteins elucidates their physiological role



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ABSTRACT

Most internationally travelled researchers will have encountered jetlag. If not, working odd hours makes most of us feel somehow dysfunctional. How can all this be linked to circadian rhythms and circadian clocks? In this review, we define circadian clocks, their composition and underlying molecular mechanisms. We describe and discuss recent crystal structures of *Drosophila* and mammalian core clock components and the enormous impact they had on the understanding of circadian clock mechanisms. Finally, we highlight the importance of circadian clocks for the daily regulation of human/mammalian physiology and show connections to overall fitness, health and disease.

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1. Introduction

1.1. Circadian clocks and physiology

Most organisms from cyanobacteria to man have developed endogenous circadian (that is about 24 h) biological rhythms [1–7] in order to adapt to the geophysical day–night cycle. Many human physiological functions and patterns of behavior underlie circadian regulation by a so-called circadian clock. Examples include sleep–wake rhythms, body temperature and blood pressure, the production of hormones or the activity of the immune system [8–11]. Additionally, a connection between circadian clocks and cell cycle regulation as well as the control of genome stability is becoming increasingly evident [12]. Disruption of circadian rhythms (e.g. due to genetic mutations, frequent jet-lag or shift-work) is linked to diverse pathogenic processes such as sleep disorders, depression, metabolic syndrome, obesity, cardiovascular diseases and even carcinogenesis [13–15] (Fig. 1A). Chronobiology arose as a scientific field to analyze these increasingly complex correlations and this subsequently led to the development of chronotherapy as a clinical application [16–18]. Furthermore, clock modulating compounds have been identified [19,20] and their potential use in the treatment of clock related and metabolic disorders is under exploration.

Circadian clocks and rhythms are synchronized to the environment by external stimuli (Zeitgeber signals), most importantly the light–dark cycle of a day's period, but also by temperature. In mammals, the central oscillator (*Pacemaker/Masterclock*) is located in the suprachiasmatic nucleus (SCN) of the brain's hypothalamus. Additionally, mammals possess peripheral clocks in nearly all of their organs and tissues to generate local circadian rhythms in physiology [21]. The central oscillator in the SCN is synchronized to the environmental light–dark cycle via photic cues, which are detected by the circadian photoreceptor melanopsin in the retinal ganglion cells [22] and are transmitted to the SCN via the optic nerve. In the SCN neurons, phosphorylation of the transcription factor CREB activates the transcription of *per1* and *per2* clock genes [23,24]. This in turn serves as an input to the main transcriptional feedback loop underlying the cell-autonomous molecular circadian oscillator (see below). Neuronal and humoral output signals of the SCN are perceived as input signals by peripheral body clocks to orchestrate circadian physiology and behavior in synchrony with the environment [21,15]. Different from the SCN master clock, peripheral body clocks can also be synchronized by feeding rhythms as dominant Zeitgeber signals. Irregular feeding rhythms may therefore lead to an uncoupling of peripheral clocks from the SCN master clock, which can affect fitness and even health. Notably, in our modern society, irregular food intake frequently arises from unusual rest–activity cycles due to shift work or jetlag [25,13]. As we will discuss below, the mammalian molecular clockwork provides input pathways to sense the cells metabolic, redox and energy states

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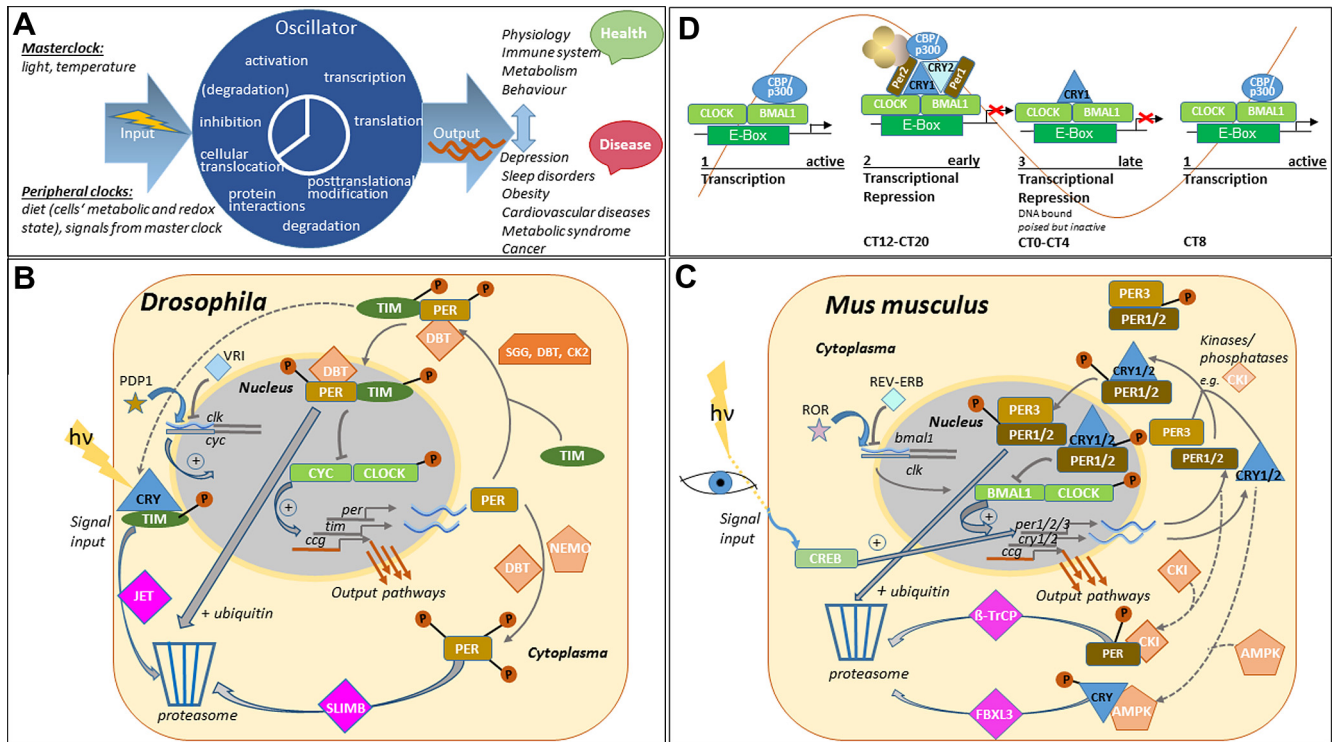


Fig. 1. Circadian physiology and molecular clocks. (A) Schematic description of the circadian oscillator and its input and output pathways. The central oscillator (master clock) time is set predominantly by light but also by temperature as environmental input signals (Zeitgeber). Oscillators in peripheral tissues (peripheral clocks) depend on metabolic steering and signals of the master clock. The input signal is integrated by the core oscillator, which is operated by cell-autonomous transcriptional and translational feedback loops. The endogenous 24 h period arises from the timely coordinated activation and repression of transcription, protein synthesis and degradation, posttranslational and posttranscriptional modifications, protein interactions and changes in cellular localization. Output signals derived from clock controlled gene activation (e.g. metabolic proteins, hormones) define circadian physiology and behavior as well as health balance. Imbalanced and dysfunctional states can lead to diseases like metabolic syndrome, obesity, diabetes, cardiovascular disease, cancer, depression and sleep disorders. (B) Molecular oscillator of *Drosophila melanogaster*. The main feedback loop including CLOCK (CLK) and CYCLE (CYC) as transcriptional activators and PERIOD (PER) and TIMELESS (TIM) as repressors is shown. In a second loop, VRI and PDP1 regulate the daily synthesis of CLOCK. Phosphorylation of PER and TIM (by kinases SGG, DBT, CK2, NEMO) as well as PER-TIM-(DBT) complex formation regulates their stability and cellular localization. The E3 ligases SLIMB and JETLAG (JET) mediate PER and TIM proteasomal degradation. TIM degradation is triggered by light-dependent interactions with the photoreceptor Cryptochrome (CRY). (C) Molecular oscillator of *Mus musculus*/mammals. The main feedback loop including CLOCK (CLK) and BMAL1 as transcriptional activators and PERIOD 1,2,3 (PER1,2,3) and Cryptochrome 1,2 (CRY1,2) as repressors is shown. In a second loop, REV-ERB and ROR regulate the daily synthesis of BMAL1. Phosphorylation of PERs and CRYs (only kinases CKI and AMPK are shown to reduce complexity) regulates their stability and cellular localization. BMAL1/CLOCK phosphorylation affects their transcriptional activity. E3 ligases TrCP1/2 and FBXL3 mediate proteasomal degradation of PER and CRY. PER1/2-CRY1/2 complexes as well as homo- and heterodimeric PER(1-3)-PER(1-3) complexes can be formed. (D) Daily transcriptional repression and activation cycles of the mammalian circadian clock. (1) In a transcriptionally active state CLOCK/BMAL1 are bound to the E-box DNA and BMAL1 recruits CBP/p300. (2) In the early transcriptional repression phase (about CT12–CT20), large complexes including CRY, PER and other factors, which affect chromatin structure and transcription termination (empty bulks), are recruited to BMAL1/CLOCK. (3) During the late transcriptional repression phase (about CT0–CT4) a repressive DNA-bound BMAL1/CLOCK/CRY complex is formed. (4) Dissociation of CRY and recruitment of CBP/p300 leads to reactivation of transcription at about CT8. CT = Circadian time; CT 0 = sunrise/light on/activity onset in diurnal organisms; CT 12 = sunset/light off. Fig. 1D was adapted from [46].

and their changes upon feeding and fasting. These input pathways are sensitive e.g. to changes in cellular levels of NAD^+ or ATP.

1.2. Molecular circadian clocks of *Drosophila melanogaster* and mammals

In the model organisms *D. melanogaster* and mouse, that we want to focus on in this review, the circadian oscillators have been extensively analyzed at molecular level [26,6,7]. They are operated by cell-autonomous gene-regulatory feedback loops with an endogenous free-running period of roughly 24 h. The molecular oscillators are composed of transcriptional activators and repressors. Additionally, a number of proteins (e.g. kinases, phosphatases, E3 ubiquitin ligases, (de)acetylases) post-translationally modify their target proteins and thereby periodically regulate their transcriptional activity, cellular localization, interactions, stability and cellular concentration. Physiological outputs are generated by the regulation of clock controlled genes (ccgs), which constitute about 10% of all genes and vary in a tissue-specific manner. In the following, key elements of the *Drosophila* and mammalian circadian oscillators will be described (Fig. 1B and C).

1.2.1. The *Drosophila* circadian oscillator

In the main gene regulatory feedback loop of the *Drosophila* circadian oscillator (Fig. 1B), the basic helix-loop-helix (bHLH) PAS (PERIOD; ARNT; SINGLEMINDED) transcription factors dCLOCK (dCLK) and dCYCLE (dCYC) form a heterodimer, which binds to the E-box (enhancer box) promoter region of the clock genes *period* (*per*, dPER) and *timeless* (*tim*, dTIM) and thereby activates their expression [26,6]. The concentration of newly synthesized dPER and dTIM proteins stays low to begin with, since protein accumulation is delayed by their proteasomal degradation. dPER degradation is initiated by phosphorylation through DOUBLETIME (DBT), a homolog of casein kinase I (CKI), which leads to the recruitment of the E3 ubiquitin ligase SLIMB. DBT phosphorylation in turn is primed by the kinase NEMO [27–29]. After sunset (around circadian time (CT) 12; see glossary for definition of CT), the cytosolic concentrations of dPER and dTIM reach their highest level. dTIM binds to the dPER-DBT complex and thereby stabilizes dPER. Phosphorylation of dTIM by SHAGGY (SGG), a homolog of glycogen synthase kinase 3 (GSK3), as well as dPER phosphorylation by casein kinase 2 (CK2) promote nuclear entry of PER-TIM-DBT complexes. In the nucleus, dTIM and dPER associate with and repress

the transcriptional activity of the dCLK:CYC heterodimer. Thereby, dPER and dTIM inhibit the expression of their own genes as well as of *cogs* affecting the flies' behavior, metabolism and development [26,6]. Light-synchronization of the endogenous clock is mediated by the circadian photoreceptor *Drosophila* cryptochrome (dCRY). Upon light-exposure, dCRY undergoes a conformational change enabling dTIM binding, which is followed by dTIM proteasomal degradation via the E3 ubiquitin ligase JETLAG (JET) [30,31]. As dTIM is sequestered from the feedback loop, the remaining free dPER protein also is degraded following phosphorylation by DBT. In that way, transcriptional repression is abolished and newly synthesized dCLK can interact with continuously expressed dCYC to activate transcription, which will restart the circadian cycle. The daily rhythmic dCLK synthesis is controlled by a second interconnected feedback loop, in which the basic leucine zipper (bZIP) transcription factors PAR DOMAIN PROTEIN1 (PDP1) and VRILLE (VRI) act as positive (PDP1) and negative (VRI) regulators of *dclk* gene expression. The *vri* and *pdp1* genes themselves are regulated by dCLK/CYC [26,6].

1.2.2. The mammalian circadian oscillator

In the main feedback loop of the mammalian circadian oscillator (Fig. 1C), the heterodimeric bHLH-PAS transcription factors BMAL1 and CLOCK (homologs of dCYC and dCLK) activate the transcription of three period (*mper1,2,3*) and two cryptochrome (*mcr1,2*) genes as well as many clock controlled genes (*cogs*) that determine circadian physiology and behavior [2,7]. Opposite to dCLK and dCYC, mammalian BMAL1 is rhythmically expressed, whereas CLOCK is continuously produced. Rhythmic BMAL1 expression is ensured by a second interconnected feedback loop, in which two nuclear receptors, REV-ERB and ROR, repress (REV-ERB) or activate (ROR) BMAL1 transcription. The *rev-erb* and *ror* genes are also targets of BMAL1/CLOCK. Different from dCRY, the mCRY proteins act as potent and light-independent transcriptional repressors of the BMAL1/CLOCK complex. Hence, mammalian CRYs functionally replace *Drosophila* Timeless (dTIM) as transcriptional repressors and binding partners of the PER proteins. mCRY1^{-/-} knockout mice display a 1 h shorter and mCRY2^{-/-} knockout mice a 1 h longer period, while mCRY1/CRY2 double knockout mice are totally arrhythmic [32–34]. Hence, mammalian CRY1 and CRY2 are partially redundant, but also have non-redundant clock functions. The mCRY repressor activity is determined by posttranslational modifications (e.g. mCRY phosphorylation) as well as the daily rhythmic synthesis, degradation and nuclear translocation of the mCRYs, which are controlled by interactions with the E3 ubiquitin ligase FBXL3 and the mPER1/2 proteins. Interestingly, mCRY1 phosphorylation at Ser71 by AMPK reduces mCRY1 stability by enhancing FBXL3 binding and weakening mPER2 binding [35]. Since AMPK activity is regulated by the AMP/ATP ratio in the cell, this provides a potential link between the cells metabolic and energy state and the circadian clock. Amongst the three mammalian PERIOD orthologues, mPER1 and mPER2 constitute essential time keeping elements of the central SCN master clock, whereas mPER3 only has a subtle effect on the generation of behavioral rhythms [36]. Instead, mPER3 appears to play a role in peripheral clocks and output functions, since mPER3 disruption and human PER3 polymorphisms lead to aberrant metabolic and sleep phenotypes [37,38]. Apart from regulating mCRY stability and cellular localization, mPER proteins participate in transcriptional regulation of BMAL1/CLOCK by multifaceted mechanisms (discussed in detail below, Fig. 1D). Mammalian PERs are phosphorylated by casein kinase I (CKI), which affects their stability, protein interactions and nuclear translocation. Additionally, casein kinase 2 (CK2) collaborates with CKI in mPER2 degradation [39], and phosphorylation by GSK-3 β promotes mPER2 nuclear translocation [40]. CKI-dependent

proteasomal degradation of mPER1 and mPER2 is mediated by the E3 ubiquitin ligase β -TrCP (β -transducin repeat-containing protein), the homolog of SLIMB [41,42]. Additionally, mPER2 [43] and BMAL1 [44] are acetylated and their deacetylation is carried out by Sirtuin1 (SIRT1). As SIRT1 is an NAD⁺ dependent enzyme, the regulation of its enzymatic activity may provide an input pathway for the clock to sense the cellular NAD⁺ concentration and hence the cell's metabolic and redox state.

Notably, there is a significant and constantly increasing number of additional molecular processes discovered that modulate the pace of the *Drosophila* and mammalian circadian clock, including e.g. further posttranslational modifications or posttranscriptional effects, which we do not discuss because their complexity would go far beyond the scope of this review. Instead, let us move on to this review's main intention: to present recent crystal structures of *Drosophila* and mammalian clock proteins, the insights they provided and the follow-up experiments they inspired.

2. Structural insights into *Drosophila* and mammalian circadian clock regulation

2.1. PAS domains in PERIOD, CLOCK and BMAL1 clock proteins as versatile interaction modules

The timely interaction of PAS (PERIOD-ARNT-SIM) domains and LOV (Light-Oxygen-Voltage) domains (a subclass of sensory PAS domains) is a recurring feature of circadian clock regulation. PAS and LOV domains are present in transcriptional activator and repressor proteins in the main gene regulatory feedback loop of the fungal, fly and mammalian circadian oscillators. Additionally, they are present in the plant ZTL/FKF1/LKP2 photoreceptor family, that tunes circadian rhythms and photoperiodic flowering [45–47]. Apart from their dominant presence in circadian clock systems, PAS and LOV domains have been identified in a large number of signaling proteins mediating protein interactions or sensing environmental signals such as light, redox potentials or the presence of oxygen [48].

PAS domains consist of 120–150 residues arranged in a sheet of five antiparallel β -strands (herein referred to as β A – β E) and flanking α -helices (herein referred to as α A – α C) (Fig. 2). The helical segments pack across one face of the β -sheet forming a pocket that frequently accepts ligands or sensory cofactors such as heme, FMN, FAD, metabolites or divalent cations. In many PAS and LOV domains, the remaining PAS β -sheet surfaces are covered by additional elements (N-terminal caps or α -helices) located N- or C-terminal to the PAS core domain, providing structural and functional diversity to the overall theme [49,50]. PAS and LOV domains are frequently coupled to other signaling modules such as histidine- or serine/threonine kinases, phosphodiesterases, ion channels or transcription factors, whose activity they modulate in dependence of the signal they are able to sense [50].

Drosophila and mammalian PERIOD (d/mPER) clock proteins as well as the mammalian bHLH-PAS transcription factors CLOCK, NPAS2 and BMAL1 and their *Drosophila* homologs dCLOCK (CLK) and dCYCLE (CYC) possess two PAS domains organized in tandem, referred to as PAS-A and PAS-B (Fig. 2). In the PERIOD proteins, the PAS domains mediate homodimer interactions, which likely modulate the functionality of more C-terminal regions of the molecule. The latter include motifs that control the cellular localization or stability of the PER proteins, posttranslational modification sites, regions involved in transcriptional regulation (e.g. the dCLK/CYC inhibition domain (CCID) of dPER), binding sites for other clock components (e.g. CKI ϵ or CRY) as well as binding sites for molecules that mediate physiological functions (e.g. nuclear receptors). The PAS domains of the bHLH-PAS transcription factors mediate

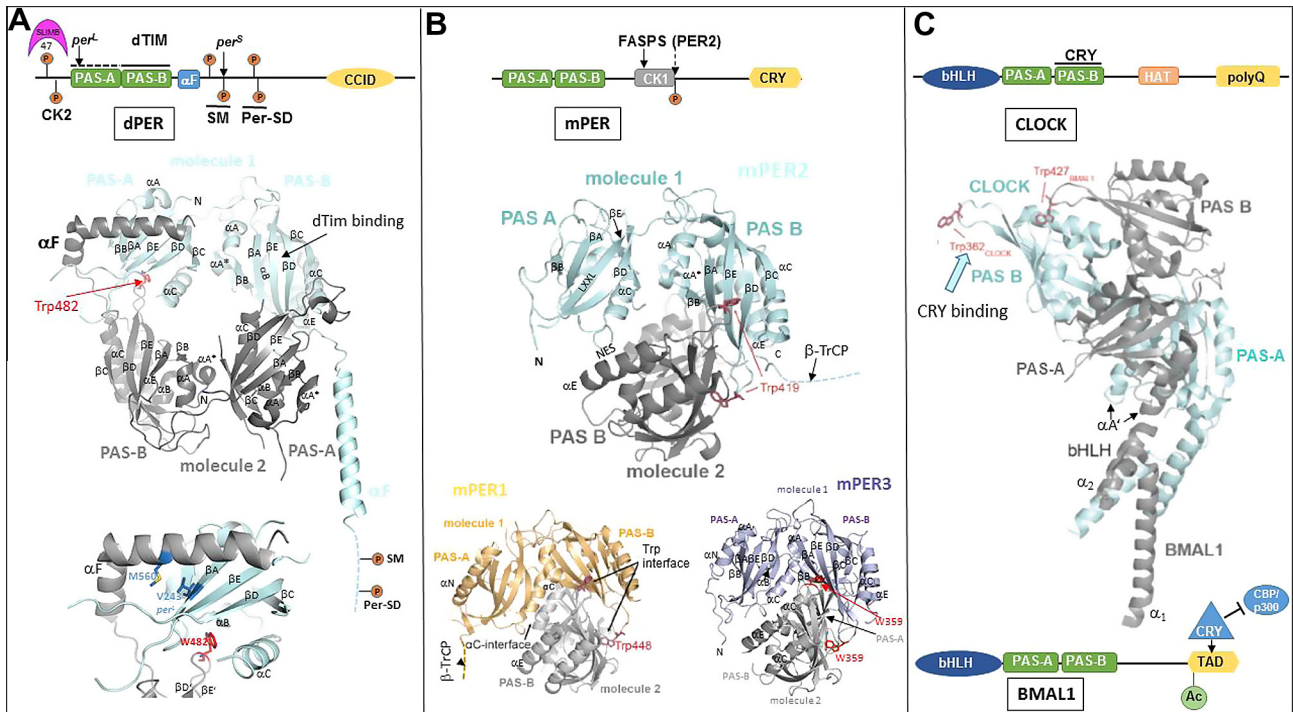


Fig. 2. PERIOD, CLOCK and BMAL1: PAS domains and their versatile interplay. (A) Domain organization and crystal structure of *Drosophila* PERIOD (dPER) *Top*: domain schematic of dPER. Two PAS domains (PAS-A and PAS-B) are N terminally preceded by CK2 and DBT phosphorylation sites. The latter contains Ser47, which (when phosphorylated) mediates interaction with SLIMB for dPER degradation. The PAS-B domain predominantly binds dTIM (TIMELESS). The PAS-A domain also interacts with dTIM and contains a long period mutation site (*per^L*). A short period mutation site (*per^S*) lies within the SM region, which is followed by the Per-SD (per-short downstream) region. The SM and per-SD regions are extensively phosphorylated and can affect Ser47 phosphorylation. The CCID (dCLK:CYC inhibition domain) is involved in transcriptional repression. *Middle*: Crystal structure of the dPER homodimer [56]. The dPER homodimer is stabilized by PAS-A-PAS-B interactions via the conserved Trp482. Additionally, helix α F C-terminal to PAS-B binds to PAS-A of the dimerizing molecule. Helix α F adopts different conformations in the two dPER monomers. The SM- and Per-SD regions are directly C-terminal to α F. dPER homodimer formation and changing α F conformations may therefore affect dPER stability by modulating phosphorylation efficiency. *Bottom*: Close-up view of the PAS-A- α F homodimer interface. The *per^L* (Val243Asp) mutation site and Met560 in helix α F opposite Val243, which was used for mutational analyses of dPER homodimer functions (Met560Asp mutation) [66], are shown as atomic stick figures. (B) Domain organization and crystal structures of mouse PERIOD 1,2,3 (mPER 1,2,3). *Top*: domain schematic of mPER proteins. Two PAS domains (PAS-A and PAS-B) are C-terminally followed by a CKI binding and phosphorylation site. This region also contains the FASPS (familial advanced sleep phase syndrome) mutation site. The mammalian CRYs bind to the C-terminal region of the mPERs. *Middle and bottom*: Crystal structures of mPER2 (middle), mPER1 and mPER3 (bottom) homodimers. mPER homodimers are mediated by antiparallel interactions of the PAS-B β -sheet surfaces including the conserved tryptophans Trp419 (mPER2), Trp448 (mPER1) and Trp359 (mPER3) corresponding to Trp482 of dPER. In the mPER2 structure, a nuclear export signal (NES) in the α E helix, a nuclear receptor binding motif (LxxLL) in the β E-strand of PAS-A, and a TrCP E3 ligase binding site for mPER2 degradation located C-terminal to α E are labeled. In mPER1, the β -TrCP interaction site is located N-terminal to PAS-A. mPER1 is stabilized by an additional PAS-A interface mediated by the α C helices and has a well-structured N-terminal cap helix (α N). These features (α C interface, N-terminal cap) are partly also observed in mPER3, but are absent in mPER2. Adapted from [59,58] and the PhD thesis of Ira Schmalen (2014). (C) Domain organization and structure of the CLOCK-BMAL1 heterodimer. *Top and bottom*: domain schematics of CLOCK (top) and BMAL1 (bottom). CLOCK contains a N-terminal E-box-DNA binding bHLH (= basic Helix-Loop-Helix) domain, PAS-A and PAS-B domains, an unconventional HAT (= histone acetyl transferase) domain and a polyQ (= poly glutamine) domain. The PAS-B domain of CLOCK is reported to interact with cryptochromes. BMAL1 contains a bHLH domain, PAS-A and PAS-B domains and a C-terminal transactivation domain (TAD), which interacts with mCRY in an acetylation-dependent manner or with p300/CBP. *Middle*: Crystal structure of the CLOCK-BMAL1 heterodimer [54]. CLOCK and BMAL1 are arranged in parallel such that each module (bHLH, PAS-A, PAS-B) interacts with its counterpart in the dimerization partner. The conserved Trp427 in PAS-B of BMAL1 interacts with PAS-B of CLOCK, while the conserved Trp362 of CLOCK provides a binding site for mCRY. The PAS-A and -B domains are arranged as in the mPER monomers in BMAL1, but differently in CLOCK. Adapted from PhD thesis of Ira Schmalen (2014).

dimer formation and partner specificity [51] and thereby define their DNA targets, transcriptional activity and transcriptional regulators to be recruited. These functions in turn are mediated by the N-terminal E-box-DNA binding bHLH domain or by transcriptional activation domains located C-terminal to the PAS domains. Mammalian CLOCK for example has a C-terminal glutamine rich activation domain preceded by a histone acetyl transferase (HAT) like domain, which is reported to acetylate Lys537 of its heterodimerization partner BMAL1 in a daily rhythmic manner [52] as well as histone H3 Lys14 [53]. BMAL1 Lys537 acetylation enhances the recruitment of mCRY1 to the C-terminal region of BMAL1 and thereby transcriptional repression [52]. Additionally, mCRY1 is reported to bind to the PAS-B domain of CLOCK [54]. Release of the mCRY1 repressor from the BMAL1/CLOCK complex allows for the binding of the HAT CBP/p300 to overlapping C-terminal BMAL1 regions, which leads to transcriptional activation in a timely manner [55,46].

During the past decade, crystal structures of PAS domain fragments of *Drosophila* PERIOD (dPER) [56,57] and the mouse

PERIOD homologs mPER1,2,3 [58,59] have been determined. Furthermore, 3D structures of the bHLH-PAS domain regions and the E-Box-DNA bound bHLH-domains of the mammalian CLOCK/BMAL1 complex were reported [60,61]. These structures shed light on the organization of the tandem PAS-A and -B domains within PERIOD homodimers and the CLOCK/BMAL1 heterodimer as well as suggest possible consequences of PAS-mediated dimerization on molecule functions. While the domains themselves adhere to standard PAS architecture, their arrangements within the dimers are remarkably different (Fig. 2). In the following, results and important biological insights gained from these clock protein crystal structures will be discussed.

2.2. Structure and function of the dPER PAS domain homodimer

In the dPER homodimer structure, the PAS domains are arranged in a head to tail configuration such that PAS-A interacts with PAS-B of the opposing subunit [56] (Fig. 2A). A loop of the PAS-B β -sheet (connecting strands β D and β E) inserts a highly

conserved tryptophan (Trp482) into the hydrophobic pocket of PAS-A of the dimerizing molecule. A second homodimer interface is generated by an additional α -helix C-terminal of PAS-B named α F, which – in its kinked conformation – packs against the PAS-A β -sheet surface of the dimerizing subunit [56]. Notably, this α F helix is highly dynamic as it can associate with PAS-A with varying helical registers [57] and also can adopt a stretched conformation to completely dissociate from the PAS-A domain of the dimerizing molecule [56]. Conformational dynamics of the α F helix are likely to influence the functionality of C-terminal regions of dPER including phosphorylation sites (see below) or the dCLK/CYC inhibition domain (CCID) [62]. Interestingly, the α F helix directly contacts the site of the *per^L* (Val243Asp) long period mutation that is located on the PAS-A- β -sheet surface within the PAS-A- α F homodimer interface (Fig. 2A bottom). This mutation confers temperature-sensitive phenotypes of clock period lengthening to about 29 h [63], delayed nuclear entry of dPER [64] and reduced dTIM binding [65]. Moreover, the *per^L* mutation disrupts dPER homodimers in vitro [56]. We therefore propose that the *per^L* phenotypes are correlated with a destabilization of the dPER homodimer, which, as we discuss below, plays an important role in the fly circadian clock. While the PAS-A β -sheet is covered by the α F helix of the dimerizing molecule, the β -sheet surface of the PAS-B domain remains surface exposed (Fig. 2A). Analyses of a triple mutant on the PAS-B β -sheet surface by analytical gel filtration and yeast-2-hybrid experiments showed, that the PAS-B β -sheet surface is involved in dTIM binding, but is not required for dPER homodimer formation as the crystal structure suggests [59].

A comprehensive in vivo study with flies expressing a mutant dPER protein with a Met560Asp exchange in helix α F directly opposite the *per^L* site of the dimerizing molecule (Fig. 2A bottom), established the role of the dPER homodimer in the fly circadian clock [66]. As suggested by the dPER structure, the Met560Asp mutation reduced dPER homodimer formation in vitro and in the fly without affecting dTIM binding [59,66]. Reduced homodimer formation coincided with a somewhat (approx. 1 h) longer period length of the flies' behavioral rhythms, changes in PER nuclear translocation and phosphorylation as well as reduced repressor activity of PER toward dCLK:CYC [66]. Hence the dPER homodimer is important for circadian clock function and the phenotypes of the Met560Asp mutation are likely to arise from the weakening of the PAS-A- α F dimer interface. Interestingly, the Met560Asp mutant phenotypes mirror those of mutations in casein kinase 2 (CK2), which phosphorylates dPER on residues 149 to 151 N-terminal to PAS-A [67–70]. The more drastic period lengthening and temperature-sensitive phenotype of the *per^L* mutation in the same interface is presumably due to the fact that *per^L* not only weakens the PAS-A- α F interface and hence homodimer formation [56], but (in contrast to Met560Asp) also affects dTIM binding to PAS-A [65].

A second set of mutation sites leading to short period phenotypes with period lengths of 16 h (*per^T*, Gly593Asp) to 19 h (*per^S*, Ser589Asn) [63,64] is located just C-terminal to the flexible α F helix of dPER in a region referred to as short mutable (SM) region. Interestingly, the SM region and the C-terminally adjacent so called per-short downstream (Per-SD) region contain a number of phosphorylation sites that are critical for clock function, including the *per^S* site (Ser589) itself [29,71,72]. Sequential phosphorylation of serine residues in the SM and Per-SD regions by first the kinase NEMO and then Doubletime (DBT) ultimately inhibits the DBT dependent phosphorylation of yet another serine residue (Ser47) located N-terminal to PAS-A. Phosphorylation of Ser47 in turn is critical for the recruitment of the E3-ligase SLIMB that mediates dPER degradation (Fig. 2A) [27–29]. Hence, this phosphorylation cascade sets the clock's pace by regulating dPER stability. The dPER crystal structure suggests that conformational changes of the α F helix between the kinked (PAS-A bound) and the stretched

state (Fig. 2A) could influence or be affected by the phosphorylation state of the Per-SM and Per-SD regions, which are only 20 and 40 residues apart from the C-terminal end of the α F helix. Furthermore we hypothesize, that homodimer formation and the α F conformation could influence the efficiency of Ser47 phosphorylation and hence dPER stability [27] by modulating the distance of the C-terminal SM- and Per-SD regions to the N-terminal Ser47 phosphorylation sites. Modeling studies based on the dPER homodimer structure support this possibility and suggest that the kinked (PAS-A bound) α F conformation may be more efficient in achieving proximity of these phosphorylation sites. However, experimental proof is still required.

Notably, the arrangement of phosphorylation sites in the Per-SD region resembles that of the casein kinase I ϵ (CKI ϵ) phosphorylation motif in human PERIOD2 (hPER2), that has been implicated in the Familial Advanced Sleep Phase Syndrome (FASPS) (Fig. 2B) [71]. FASPS is an autosomal dominant circadian rhythm variant with a 4-h advance of sleep, temperature and melatonin rhythms. FASPS is caused by a Ser662Gly mutation in hPER2, that prevents CKI ϵ dependent phosphorylation [73] and thereby also affects PER2 stability and activity [73–75].

2.3. From flies to mammals: structures of the mouse PERIOD 1,2,3 PAS domain homodimers and functional insights

In mammals, three PERIOD homologs (mPER1, mPER2 and mPER3) constitute central components of the circadian clock. Their PAS domains (PAS-A and PAS-B) mediate homo- and heterodimeric mPER-mPER interactions as well as interactions with the transcription factors BMAL1/2, CLOCK and NPAS2 [76,55,77,78], with kinases (e.g. CK2 [39], GSK 3 β [40]) and with nuclear receptors of different physiological functions [79]. Heme binding to the PAS domains of mPER proteins has also been observed [80–82,58] but is controversially discussed [83].

Crystal structures of PAS domain homodimers of all three mPER homologs have been determined [59,58] (Fig. 2B). Different from the head-to-tail arrangement observed in dPER, the PAS domains of all three mPER homologs stack in an antiparallel fashion via their PAS-B β -sheet surfaces. Despite their different architecture, the mPER homodimers also include the highly conserved tryptophan (Trp448_{mPER1}, Trp419_{mPER2}, Trp359_{mPER3}) corresponding to Trp482_{dPER} as a central dimer interface residue. As in dPER [56,59,66], mutation of the conserved tryptophan affects mPER dimerization in vivo and in vitro [59,58]. Hence, the PAS-B β -sheet surface of PERIOD turns out to be a versatile interaction site which mediates mPER homodimerization in the mammalian clock and dPER-dTIM heterodimer formation in *Drosophila* [56,59]. Furthermore, modeling suggests, that mPER1/2/3 heterodimers could also be formed via this PAS-B interface.

Due to somewhat changed relative orientations of the mPER monomers, mPER1 homodimers are additionally stabilized by interactions between the PAS-A domains via their long α C helices (Fig. 2B). Furthermore, mPER1 and mPER3 have an N-terminal cap helix (α N) N-terminal to their PAS-A domains, while the corresponding region of mPER2 is unstructured. Biochemical analyses of the PAS homodimer interfaces using analytical ultracentrifugation revealed a ten times higher dimer affinity for mPER1 ($K_D = 0.15 \mu\text{M}$) compared to mPER2 ($K_D = 1.3 \mu\text{M}$) and mPER3 ($K_D = 1.7 \mu\text{M}$), likely due to the additional PAS-A homodimer interface of mPER1 [58].

A functional role of mPER homodimers in the mammalian circadian clock has been suggested by Fluorescence recovery after photobleaching (FRAP) experiments that observe the mobility of multi-subunit MDa sized mPER-CRY containing complexes in mammalian cell culture [58]. Apart from PER and CRY proteins, these multi-subunit complexes contain various transcriptional

regulators affecting e.g. chromatin structure or transcription termination [84–87]. mPER proteins are thought to contribute to transcriptional feedback repression by delivering these complexes and their intrinsic enzymatic functions to the DNA-bound BMAL1/CLOCK complex to inhibit BMAL1/CLOCK activity via multifaceted and timely regulated mechanisms [87]. Interestingly, FRAP experiments revealed, that mPER2 containing multi-subunit complexes exhibit a faster mobility upon destabilization of the PAS-B/tryptophan homodimer interface of mPER2 [58]. Hence, PER2 homodimers appear to be relevant for the integrity of these large and functionally important clock protein complexes.

Based on the relatively low homodimer affinity of its PAS domains, the higher flexibility of its PAS-A domain and the unstructured N-terminal cap, mPER2 seems to be more predisposed for heterodimeric signaling interactions than mPER1 and mPER3. Consistently, the PAS domains of mPER2 but not mPER1 have been reported to bind to casein kinase 2 (CK2) [39], glycogen synthase kinase 3 β (GSK 3 β) [40], REV-ERB and other nuclear receptors [79]. Notably, mPER2 contains an LXXLL coactivator motif typical for nuclear receptor binding in the β E strand of PAS-A, which is reported to be involved in mPER2 binding to REV-ERB α and PPAR α [79]. In mPER1 and mPER3, the corresponding motifs contain sequence exchanges and moreover they are buried underneath the well-defined N-terminal cap helices (α N) of mPER1 and 3 (Fig. 2B, [58]). These structural features may correlate with the reported inability of mPER1 to interact with nuclear receptors [79]. Interestingly, the nuclear receptor binding motif of mPER2 also plays a role in mPER2 nuclear import and nuclear immobility [88], possibly by mediating binding to nuclear receptors in the nucleus (effect on nuclear immobility) or in the cytoplasm (effect on nuclear import).

dPER and mPER proteins have an extra α E helix C-terminal to PAS-B which packs against the PAS-B core domain (Fig. 2A and B) [56,59,58] and contains a Nuclear Export Sequence (NES) in all three mPER homologs [89]. Due to the somewhat changed relative orientations of the mPER monomers, the NES is engaged in homodimer contacts in mPER2 but not in mPER1 and mPER3. Homo- and heterodimer interactions of the mPER proteins are therefore likely to modulate the function of this NES in the circadian clock. Different monomer orientations, homodimer affinities and N-terminal cap structuring may also correlate with the distinct locations of the sites for the CKI ϵ -dependent recruitment of the E3 ligases β -TrCP1/2, that are responsible for mPER degradation. The respective CKI ϵ phosphorylation sites are located N-terminal to PAS-A in mPER1 (residues 121–126, TSGCSS) and directly C-terminal to the α E helix in mPER2 (residues 477–482, SSGYGS) [41,42] (Fig. 2B).

2.4. Structure–function analyses of CLOCK/BMAL1 dependent transcriptional regulation of the mammalian circadian clock

The crystal structure of the bHLH-PAS domain region of the CLOCK/BMAL1 complex revealed a parallel head-to-head arrangement of BMAL1 and CLOCK, where each module (bHLH, PAS-A, PAS-B) interacts with its counterpart in the opposing subunit (Fig. 2C) [54]. The N-terminal DNA binding bHLH motif recognizes the palindromic E-box sequence [90]. The PAS-A domains swap their N-terminally flanking α A'-helices to interact with the β -sheet surfaces of the PAS-A domains of the respective dimerization partner, whereas the PAS-B domains of CLOCK and BMAL1 are stacked in a parallel orientation. The PAS-B β -sheet of BMAL1 contacts the helical face of the PAS-B domain of CLOCK using the conserved Trp427_{BMAL1} (corresponding to Trp482_{dPER} and Trp419_{mPER2}) as a central residue. In contrast, the β -sheet surface of the PAS-B domain of CLOCK remains surface exposed and provides a binding site for mCRY1, including again the conserved

tryptophan (Trp362 in CLOCK) as well as adjacent residues [76]. It is not clear yet, if PER proteins bind to the CLOCK/BMAL1 complex via their PAS domains directly, and reportedly mCRY1 can only associate with the DNA-bound CLOCK/BMAL1 complex in absence of PER [91,92]. However, the two PAS domains of BMAL1 are arranged as in the PERIOD monomers, suggesting that mPER/BMAL1 heterodimers with an mPER homodimer like architecture could potentially be formed. It has yet to be established whether or not such direct mPER-BMAL1 interactions do occur in the cell and if so, in which compartment and with which cellular function.

Genome-wide analyses lead to a refined picture of BMAL1/CLOCK transcriptional regulation with three temporally separated phases: (i) an early repressive state containing multi-subunit CRY-PER complexes, that is observed between circadian time (CT, see glossary) 12 and CT20 in mouse liver, (ii) a late repressive state with an inactive DNA-bound BMAL1/CLOCK/CRY complex (CT0 to CT4) and (iii) a transcriptionally active state in which BMAL1/CLOCK recruits its coactivator p300/CBP (CT8) (Fig. 1D) [93]. In this scenario, PER proteins contribute to the transcriptional regulation of BMAL1/CLOCK by multifaceted mechanisms including the recruitment of transcriptional regulators within multi-protein CRY-PER complexes [87], the regulation of BMAL1/CLOCK posttranslational modifications [94] as well as competing interactions with mCRY1/2 [95–97] and possibly BMAL1/CLOCK [78]. PER-CRY complex formation would on one hand spatially interfere with and thereby delay the formation of the late repressive DNA-bound BMAL1/CLOCK/CRY complex, but on the other hand also have a positive impact on the availability of CRY as a transcriptional repressor in the nucleus. The availability of CRY is warranted by promotion of its nuclear entry as well as by preventing binding of the E3 ligase FBXL3 and subsequent proteasomal degradation [98,96,97,99]. Competing interactions are also involved in the transition between the late repressive state and the transcriptionally active state. Here, mCRY1 and CBP/p300 compete for their common binding sites in the transcriptional activation domain (TAD) at the C-terminal end of the BMAL1 molecule (Fig. 2C) [76,55,100,101]. mCRY1 binding to BMAL1/CLOCK is thought to delay the formation of the transcriptionally active state by preventing the binding of the coactivator CBP/p300 to BMAL1 [93].

2.5. A molecular mechanism for the repressive and acetylation dependent mCRY1-BMAL1 interaction

CRYs are composed of a conserved photolyase homology region (PHR) consisting of an N-terminal α β -domain and a C-terminal all-helical domain as well as variable C-terminal extensions referred to as tails (Fig. 3A and B) [102]. The PHR binds the cofactor FAD (flavine adenine dinucleotide) in the all-helical domain and – in the related DNA repairing photolyases – a second light harvesting antenna chromophore in a secondary pocket between the all-helical and α β -subdomain [103].

In mammalian CRYs, the tails and the preceding C-terminal helix of the PHR (α 22 in mCRY1, see Fig. 3A) are involved in transcriptional repression of the BMAL1/CLOCK complex [104]. In vivo, repressive mCRY1 binding is enhanced by the acetylation of BMAL1 at Lys537, which is daily rhythmic with a peak in the early repressive phase and reportedly mediated by the heterodimerization partner CLOCK [52]. De-acetylation of BMAL1-Lys537 is mediated by Sirtuin1 (SIRT1), an NAD⁺-dependent enzyme which is regulated in a circadian manner [44].

Murine cryptochromes interact directly with the BMAL1-TAD via the conserved C-terminal helix of the PHR as well as non-conserved epitopes in the CRY1 and CRY2 C-terminal tails with K_Ds in the μ M range (Fig. 3C right, G) [105]. The CRY binding

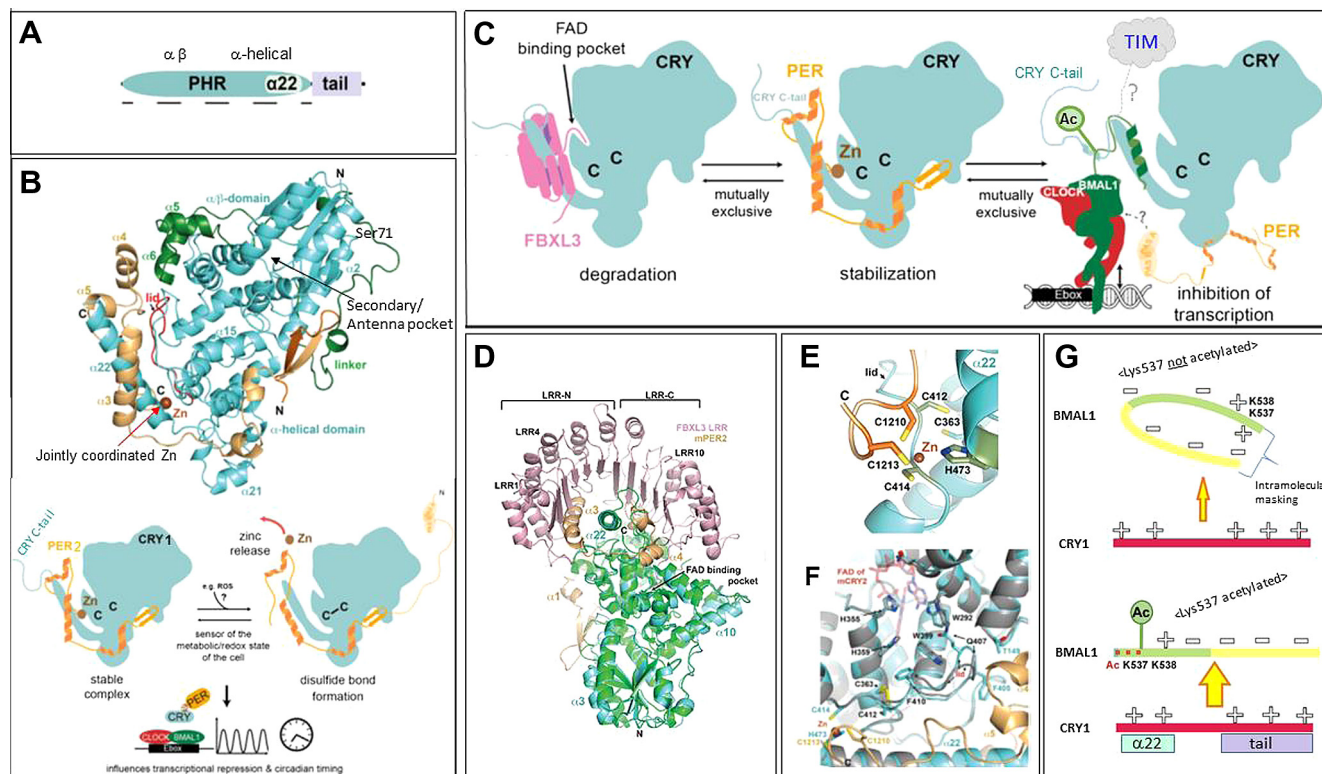


Fig. 3. Structural and biochemical analyses of mammalian cryptochrome interactions. (A) Domain organization of cryptochromes. The photolyase homology region (PHR) consists of an N-terminal $\alpha\beta$ domain and a C-terminal α -helical domain including the functionally important C-terminal $\alpha 22$ helix, which precedes the non-conserved regulatory tail. Adapted from [96]. (B) Crystal structure of the mCRY1-mPER2 complex suggests regulation by a jointly coordinated zinc ion and disulfide bond formation. Top: Crystal structure of the mCRY1-PHR in complex with a C-terminal mPER2 fragment. mCRY1 is shown in cyan, mPER2 in orange. mPER2 winds around the all-helical subdomain of mCRY1 contacting the C-terminal $\alpha 22$ helix and the lid/interface loop (red) and approaching the secondary antenna binding pocket. The mCRY1-mPER2 complex is stabilized by a jointly coordinated zinc ion (Zn). Ser71, which is phosphorylated by AMPK, is located well outside the CRY1-PER2 interface. Adapted from [96]. Bottom: Model of the suggested interplay between Zn binding and the breakage of an intramolecular mCRY1 disulfide bond upon mCRY1-mPER2 complex formation [96]. In the apo-mCRY1 crystal structure, a disulfide bond was observed between Cys412 in the lid and Cys363 near the FAD binding pocket [95]. Zn binding stabilizes the reduced mCRY1 state and thereby presumably facilitates mPER2 binding by enhancing the flexibility of the lid/interface loop (Fig. 3F). Reactive oxygen species or cellular redox equivalents could affect the formation of the disulfide bond within mCRY1 and indirectly influence the free cellular zinc concentration by affecting the redox state of zinc coordinating cysteines of zinc storage proteins. Changes in the cellular redox or metabolic state may therefore modulate the stability of the mammalian CRY-PER complex and thus transcriptional repression of CLOCK/BMAL1. The model was taken and adapted from the PhD thesis by Ira Schmalen (2014). (C) Mutually exclusive interactions of mammalian CRYs with FBXL3, PER2 or BMAL1/CLOCK regulate their stability and transcriptional repression activity. Left: Interaction with the E3-ligase FBXL3 (magenta) leads to ubiquitylation and subsequent proteasomal degradation of CRY. FBXL3 occupies the FAD binding pocket of CRY with its C-terminal tail, but also binds to the C-terminal helix of the CRY-PHR ($\alpha 22$ in mCRY1) and the lid/interface loop with its leucine-rich-repeat (LLR) domain [98]. Middle: PER2 (orange) also binds to the C-terminal helix and the lid, but does not bind in the FAD binding pocket [96,97]. Due to vastly overlapping binding sites, FBXL3 and PER2 binding to CRY are mutually exclusive. PER binding therefore stabilizes CRY by preventing FBXL3 binding. Right: The binding of PER and the CLOCK:BMAL1 complex are also mutually exclusive, because BMAL1 (green) also binds directly to the C-terminal helix of CRY [105], potentially employing a predicted BMAL1 helical segment. While the C-terminal tail of CRY is not required for PER2 binding (middle, [96]), and only partly involved in FBXL3 binding (left, [98]), it is essential for CRY-BMAL1 interactions [105]. The C-terminal tail of mCRY1 appears to sense the acetylation state of BMAL1 [105]. Furthermore, PER proteins may interact with BMAL1, CLOCK or the BMAL1/CLOCK complex via their PAS domains, suggesting another level of potential binding site competition. Moreover, mammalian TIMELESS (TIM) is reported to also interact with CRY via the C-terminal helix [145]. Adapted from PhD thesis by Ira Schmalen (2014). (D) Superposition of the mCRY1-mPER2 and mCRY2-FBXL3 complex crystal structures. Due to their vastly overlapping binding sites, binding of FBXL3 (magenta) and mPER2 (orange) to mCRY1 is mutually exclusive [98,96]. mCRY1 (cyan) and mCRY2 (green) adopt practically identical conformations in their complexes. Figure taken from [96]. (E) Close-up view of the zinc interface of the mCRY1-mPER2 complex. The zinc ion is jointly coordinated by Cys1210 and Cys1213 of mPER2 (orange) and Cys414 and His473 of mCRY1 (cyan). Cys412 of mCRY1, which is located in the vicinity of the zinc interface, forms a disulfide bond to Cys363 in apo-mCRY1 [95], but not in the mCRY1-mPER2 complex. Figure taken from [96]. (F) Close-up view of the FAD binding pocket of the mCRY1-mPER2 complex. The FAD-binding pocket, the lid/interface loop and the zinc interface of the mCRY1-mPER2 complex (cyan/orange) are shown. The apo-mCRY1 structure (gray, [95]) and FAD from the mCRY2-FAD complex (red, [98], PDB entry ID 4I6G; mCRY2 protein not shown for clarity) are superimposed. Upon mPER2 binding, the lid undergoes a conformational change. The Cys412-Cys363 disulfide bond is observed in the apo-mCRY1 structure, but not in the mCRY1-mPER2 complex. Figure taken from [96]. (G) Regulation of mCRY1-BMAL1 interaction by acetylation of Lys537^{BMAL1}. Top: In its non-acetylated state, Lys537 masks negative charges in BMAL1 through intramolecular interactions and thereby interferes with mCRY1 binding. Bottom: Upon acetylation of Lys537, BMAL1 adapts an open conformation rendering its negative charges accessible for electrostatic interactions with positively charged residues predominantly in the mCRY1 tail region. Therefore, the interaction of mCRY1 with acetylated BMAL1 is stronger than with non-acetylated BMAL1. Adapted from [105].

region of BMAL1 also includes a predicted α -helix, which may interact with the C-terminal helix of the CRY-PHR (Fig. 3C right). Interestingly, an acetyl-mimetic mutation of Lys537 to glutamine (Lys537Gln) increases the affinity of BMAL1 to the mCRY1 tail, but not to the mCRY2 tail or the C-terminal CRY helix [105] suggesting that the tail of CRY1 specifically senses the BMAL1 acetylation state. Distinct BMAL1 interactions of the CRY1 and CRY2 tails may, apart from their deviating sequences, also arise from the fact

that the CRY2 tail is phosphorylated within the BMAL1 binding epitope [106]. It will be interesting to find out, how these unique features of the CRY1 and CRY2 tails correlate with the non-redundant physiological roles of the two mammalian CRY homologs suggested by knock-out studies [32–34].

Notably, CRY1 binding to the overall negatively charged C-terminal BMAL1 TAD fragments appears to depend on electrostatic interactions with positively charged CRY1 residues

(Fig. 3G). We propose that in its non-acetylated state Lys537 masks negative charges in BMAL1 through intramolecular electrostatic interactions. Lys537 acetylation may therefore enhance CRY1 binding by unmasking negatively charged BMAL1 residues for interactions in particular with positively charged residues in the regulatory CRY1 tail epitope [105]. Notably, the presence of the PHR increases the affinity of mCRY1 to BMAL1 about ten times compared to mCRY1 fragments that only include the C-terminal helix of the PHR and the tail [95]. Circular dichroism (CD) spectroscopy suggests that the PHR leads to an enhanced structuring of the CRY1 tail, which likely contributes to the increased BMAL1 affinity. However, the PHR does not appear to affect the influence of the acetyl mimetic Lys537Gln mutation on CRY1 binding [105,95]. It remains to be investigated whether the PHR of CRY1 also interacts with the BMAL1 TAD directly.

2.6. mCRYs as transcriptional repressors – from structure to function

Having discussed the tails of the mammalian cryptochromes before their PHR core domains, we have technically put the cart before the horse. Are the PHR core domains really only there to stabilize or structurally reorganize the CRY tails? Certainly not. Despite their high degree of sequence and structural conservation also with respect to the related DNA repairing photolyases [107,103], distinct variations between the PHRs define the different roles of CRYs as blue-light photoreceptors (e.g. in insects and plants), transcriptional repressors (e.g. in vertebrates) [102] or as magnetoreceptors [108]. CRY-dependent magnetoreception has e.g. been reported in migratory birds [109,110], in the migratory monarch butterfly *Danaus plexippus* [111], in *D. melanogaster* and in plants [112,113]. Different CRY functions also correlate with their varying affinities to the FAD cofactor. While photoreceptor CRYs stably incorporate the FAD cofactor for blue-light absorption [114,115], the affinity of FAD to purified mammalian CRYs is much lower (in the micro molar range) [98], likely due their more open FAD binding pocket [95,98]. However, the crystal structure of FAD-bound mouse CRY2 showed, that FAD binds to CRY2 in a practically identical position as in plant and *Drosophila* 6–4-photolyases and photoreceptor type cryptochromes (Fig. 3F) [98,116–119,95,120]. Although mammalian CRYs have been shown to undergo photoreduction and to display light-dependent magnetoreceptor functions in a cellular environment [121,122], the biological role of FAD in the context of the mammalian CRYs is under debate. Interestingly, the crystal structure of the CRY2-FBXL3-SKB1 complex revealed that the C-terminal tail of the E3 ligase FBXL3 binds to the FAD binding pocket of CRY2 [98]. Moreover, a period-lengthening carbazolic compound (KL001) was identified in a chemical screen, which competes with FAD binding and stabilizes CRY1 by inhibiting ubiquitination [20]. The co-crystal structure of the CRY2-PHR with KL001 revealed that this compound indeed binds to the FAD binding pocket overlapping with the isoalloxazine ring of FAD as well as the FBXL3 C-terminal tail [123]. Thereby KL001 is thought to act as a mimetic of the FAD cofactor that interferes with FBXL3 binding and hence proteasomal degradation of CRY.

In addition to the interactions of the FBXL3 tail in the FAD binding pocket, the leucine-rich repeat (LRR) domain of FBXL3 associates with the C-terminal helix of the CRY-PHR and with a loop referred to as interface loop or C-terminal lid, which is located between the FAD binding pocket and the C-terminal helix (Fig. 3C left, D) [98]. The crystal structures of CRY1 [96] and CRY2 [97] in complex with a C-terminal CRY-binding PER2 fragment revealed that PER2 covers extensive surface regions of the CRY PHR including also the C-terminal helix and the lid (Fig. 3B and C middle). Hence, PER2 and FBXL3 binding to CRY is mutually exclusive due to their vastly overlapping binding sites

(Fig. 3D). PERIOD proteins can therefore stabilize CRYs by protecting them from FBXL3 binding and subsequent proteasomal degradation. As we discussed earlier, the C-terminal helix of CRY1 and CRY2 is also involved in direct interactions with the BMAL1 TAD and in transcriptional repression [105,95], suggesting an additional level of binding site competition between PER and the BMAL1/CLOCK complex (Fig. 3C right, G). Different from FBXL3, PER2 does not occupy the FAD pocket, implying that FAD could still bind to the PER-CRY complex (Fig. 3F). As suggested by the high level of conservation of the CRY-PER interface residues, almost identical interactions are observed in the CRY1-PER2 and CRY2-PER2 complex structures [96,97]. Notably, neither FBXL3 nor PER2 bind nearby Ser71 (Ser89 in CRY2) [98,96,97] implying that Ser71 phosphorylation by AMPK affects PER2 and FBXL3 interactions and thereby CRY stability [35] by an indirect mechanism. Mutational analyses of CRY in and around the secondary pocket that binds the antenna chromophore in DNA repairing photolyases showed, that this part of the CRY-PHR affects PER2 and FBXL3 binding as well as BMAL1/CLOCK transcriptional repression activity in mammalian U2OS or HEK293 cells. Further, it impacts on circadian bioluminescence rhythms in mouse embryonic fibroblast (MEF) cells [95,97,124]. In mammalian CRYs, this molecule region therefore appears to play light and presumably cofactor independent roles in circadian clock regulation, likely by affecting CRY stability and transcriptional repression activity. While PER2 approaches the secondary pocket with somewhat different conformations in the CRY1 and CRY2 complex structures [96,97], FBXL3 does not [98].

Interestingly, the CRY1-PER2 and CRY2-PER2 complexes are both stabilized by a zinc ion that is jointly coordinated by two cysteine residues of PER2 (Cys1210, Cys1213) as well as a histidine and a cysteine residue of CRY (His473 and Cys414 in CRY1) (Fig. 3B, C and E) [96,97]. Mutation of any of the zinc coordinating residues significantly weakens CRY1-PER2 complex formation in mammalian cell culture by reducing the incorporation of zinc [96]. Moreover, transgenic mice which constitutively overexpress CRY1 with a Cys414Ala mutation in the zinc interface, show a 28 h long free-running period of locomotor activity, abnormal entrainment behavior and symptoms of diabetes including reduced β cell proliferation and insulin secretion (hypo-insulinemia) [125–127]. Hence, zinc-dependent CRY1-PER2 complex formation appears to be important for circadian dynamics and metabolic regulation. Since PER binding to the C-terminal CRY helix is expected to interfere with CRY binding to BMAL1 (see above), it is conceivable, that weakening of the CRY-PER interaction by disruption of the zinc interface enhances the repression activity of the CRY(Cys414Ala) mutant protein toward BMAL1/CLOCK. This interpretation is consistent with the results of circadian rescue experiments in CRY deficient MEF cells, where the Cys414Ala mutation leads to a blunted bioluminescent rhythm amplitude of a Per-dLUC reporter gene [97]. Furthermore, the metabolic phenotypes of the Cys414Ala mutant mirror those of BMAL1 and CLOCK mutations that weaken the transcriptional activators and also lead to hypo-insulinemia and tissue-specific insulin resistance [128].

In the apo-CRY1 crystal structure, Cys412 adjacent to the zinc coordinating Cys414 in the lid/interface loop forms an intramolecular disulfide bond to Cys363 located near the FAD binding site, which is broken in the CRY1-PER2 complex structure (Fig. 3E and F) [95]. Despite its location in the reducing cytosolic and nuclear cellular compartments, CRY1 indeed also forms disulfide bonds in mammalian cells, suggesting that the Cys412-Cys363 disulfide bond may play a role in vivo [96]. Comparison of the apo-CRY1 and CRY1-PER2 complex structures suggests that reduction of the disulfide bond facilitates PER2 binding by enhancing the flexibility of the lid allowing for the conformational changes that

this loop undergoes upon PER2 binding (Fig. 3F) [95,96]. Somewhat unexpectedly, the individual mutations of the disulfide bond forming cysteines of CRY1 neither affected transcriptional repression activity [97] nor PER2 binding. However, their combination with mutations of zinc coordinating cysteines partially rescued the effect of the loss of the zinc ion on CRY1–PER2 interaction strength in mammalian cell culture [96]. This observation led us to propose that zinc stabilizes the CRY–PER complex not only by providing an extra dimer interface but also by facilitating reduction of the Cys412–Cys363 disulfide bond that anchors the lid to the FAD binding pocket. This hypothesis is supported by LC-ESI/MS mass spectrometry performed on purified CRY1–PER2 complexes, which detected disulfide bond formation in CRY1 as a 2 Da mass difference. Zinc depletion of the CRY1–PER2 complex by addition of EDTA or mutation of zinc binding residues significantly enhances CRY1 oxidation [96]. Although Cys412 and Cys363 are conserved in CRY2 (Cys430 and Cys381 in mCRY2), no intramolecular disulfide bonds have been observed in the CRY2 crystal structures [98,97]. Instead, Cys430 of mCRY2 forms an intermolecular disulfide bond to FBXL3 in the CRY2–FBXL3 complex structure [98]. Hence, Cys412 of mCRY1 and Cys430 of mCRY2 both show enhanced redox reactivity, but display distinct redox sensitivities in the apo-CRY1 and apo-CRY2 proteins. A complete understanding of the functional roles of disulfide bond formation in CRY1 and CRY2 requires further *in vivo* and *in vitro* analyses, which will be guided by the available mammalian CRY crystal structures.

Changing cellular redox states have been shown to affect circadian rhythms in a number of organisms ranging from cyanobacteria to mammals. In cyanobacteria, the ratio of oxidized to reduced quinones is involved in clock resetting by affecting the activity of the clock protein KaiA [129]. Here, the accumulation of oxidized quinones at the onset of darkness inhibits KaiA activity and thereby phosphorylation of KaiC. In the fungus *Neurospora crassa*, reactive oxygen species (ROS) are produced in a circadian manner. Fluctuating ROS concentrations regulate the activity of the WHITE-COLLAR transcription factor complex of the *Neurospora* circadian clock and affect circadian conidiation banding [130]. In mammalian SCN slices, the relative redox state has been measured from the ratio of auto-fluorescence emissions of oxidized FAD and reduced NAD(P)H using redox fluorometry. The redox state was shown to cycle in a circadian manner in SCN slices dependent on the presence of a functional molecular clock [61]. It remains to be investigated if and to what extent free zinc concentrations vary in a circadian manner. We propose that zinc binding and disulfide bond formation in CRY1 may provide an input pathway of these parameters (cellular zinc concentration as well as redox and metabolic state) to the core circadian oscillator by regulating CRY1–PER2 complex formation and thereby CRY1 stability and transcriptional repressor activity. The nanomolar K_D determined for zinc binding to the CRY1–PER2 complex [96] is within the range of free cellular zinc concentrations [131], suggesting a regulatory potential of zinc dependent CRY1–PER2 complex formation. Interestingly, cysteine oxidation states changing in an circadian manner in the H_2O_2 scavenging peroxiredoxins have been discovered as non-transcriptional circadian oscillators, which are conserved from cyanobacteria to mammals [132–135]. Furthermore, ROS have been reported to oxidize and induce disulfide bond formation of reactive cysteine residues within reducing cellular compartments in order to regulate the activity of proteins (e.g. transcription factors, chaperones, signaling proteins) operating in oxygen defense or signal transduction pathways [136]. Changing ROS concentrations may therefore indirectly or even directly influence the oxidation state of the disulfide bond forming CRY1 cysteine residues to modulate CRY1 activity in the mammalian circadian clock. We also speculate about a role of FAD in modulating the redox state of the Cys363–Cys412 disulfide bridge in CRY1

or the CRY1–PER2 complex. Since Cys363 faces the FAD binding pocket of CRY1 (Fig. 3F), such an interplay seems possible. This would imply that in mammalian CRYs (or at least in CRY1) FAD may act primarily as a redox signaling partner/cofactor rather than as light-sensor as in photoreceptor type cryptochromes (see next section).

Finally we would like to emphasize, that zinc binding to the CRY–PER complex and disulfide bond formation in CRY1 were entirely unexpected and unpredictable findings uncovered only by determining high resolution 3D crystal structures. On one hand, this unexpected structure-based discovery demonstrates the great impact that structural biology can have and has already had on the advance of molecular circadian research. On the other hand, it shows that the field should be open for yet undiscovered and alternative input pathways that could link the cell's metabolic and redox state to the circadian clock.

2.7. Back to the flies: *Drosophila* Cryptochrome as a circadian blue-light photoreceptor

Different from the mammalian CRYs, *Drosophila* Cryptochrome (dCRY) is a blue-light sensitive circadian photoreceptor that mediates synchronization of the circadian rhythm with the environmental light–dark-cycle [137] and plays a role in magnetoreception [113]. dCRY stably incorporates FAD, and blue-light illumination leads to the conversion of oxidized FAD (FAD_{ox}, dark state) to an anionic FAD^{•−} radical [114]. A cascade of three highly conserved tryptophans (Trp342, Trp397, Trp420) has been implicated in the electron transport required for FAD photoreduction [121] (Fig. 4).

The short C-terminal tail of dCRY regulates the activity of the PHR during light-synchronization of the fly circadian clock. Blue-light illumination somehow triggers a conformational change of the regulatory tail that enables binding of dTIM as well as the E3 ligase Jetlag (JET) to the dCRY–PHR, which is prevented in the dark (Fig. 4A) [138,31]. Subsequently, dTIM and dCRY are degraded in the proteasome and thereby dTIM is sequestered from the transcriptional feedback loop (Fig. 1B) [139,140,30]. The role of FAD photoreduction and of the conserved tryptophans in the regulation of tail conformational changes and dCRY signaling is controversially discussed. Ozturk et al. [141] suggest, that FAD^{•−} rather than oxidized FAD represents the ground state and therefore a light-excited form of the anionic FAD^{•−} radical is assigned to the signaling state. However, Vaidya and colleagues [142] report correlations between the light-induced or chemical reduction of FAD and subsequent conformational changes of the regulatory tail, which are consistent with FAD_{ox} being the ground state and FAD^{•−} the signaling state leading to dTIM degradation. In any case, the *cry^b* mutation (Asp410Asn) in the FAD binding pocket severely affects the biological activity of dCRY, showing that an intact protein environment of the FAD cofactor is essential for dCRY function in light-synchronization of the circadian clock [137].

Crystal structures of full-length dCRY [119,95,120] showed that the regulatory C-terminal tail forms an α -helix that occupies the position of the photolesion in DNA repairing photolyases. However, the dCRY structure reported in 2011 [119] was determined from crystals that suffered from an unusual form of non-merohedral twinning. This led to a two-residue frame shift error in the functionally important regulatory tail and its preceding linker as well as structural changes in tail adjacent loops including the lid and other characteristic loops of the animal CRY/6–4 photolyase subfamily referred to as protrusion loop, phosphate binding loop and sulfur loop (Fig. 4C). Czarna and colleagues detected these changes because they had independently determined the 2.35 Å crystal structure of a dCRY construct with a five residue truncation in the protrusion loop (dCRY Δ loop, pdb entry 4JZY),

which lead to high quality crystals and unambiguous electron density maps [95]. Using the refined dCRY Δ loop structure as a model, they solved the structure of full-length wild-type dCRY (pdb entry 4K03) by molecular replacement [95]. The dCRY structure reported by Zoltowski et al. in 2011 [119] (pdb entry 3TVS) was superseded by a newly deposited dCRY structure (pdb entry 4GU5) reported by Levy and colleagues [120], which is in full accordance with the dCRY structures obtained by Czarna et al. [95].

Czarna and colleagues [95] validated the new insights gained from the observed structural changes in the tail and its adjacent loops by extensive mutational analyses, which clearly shows the originality and independent motivation of their work. In particular, Phe534 (instead of Trp536) was identified as a residue, which anchors the tail to the PHR and assumes the same location as the 6-4-photolysis in DNA repairing photolyases (Fig. 4C). In addition, Glu530 and Ser526 anchor the tail to the PHR through extensive hydrogen bonding networks [95] and are essential for light-induced dCRY-dTIM and dCRY-dPER interactions [143].

Structural changes in the sulfur loop locate its conserved cysteine (Cys337) next to the middle tryptophan (Trp397) and its conserved methionine (Met331) near the outer tryptophan (Trp342) of the canonical electron transport cascade (Fig. 4C) [95]. The structure therefore suggests that these electron rich cysteine and methionine residues could gate the electron transfer via the tryptophan cascade and thereby slow down the rate of FAD $^{\circ-}$ formation and decay. This is conceivable, since electron flux occurs along potential slopes, which are less steep within a cascade that is affected by polarizable negative charges originating from close-by cysteines or methionines. Mutational analyses support this model showing that alanine substitution of Cys337 (near Trp397) or Cys416 (near Trp420) indeed shortens the lifetime of the anionic FAD radical. Moreover, correction of the 2-residue frame shift error places Cys523 in the tail connector loop proximal to Met421, which is also located close to Trp397 (Fig. 4C). As observed for Cys337, the Cys523Ala mutation significantly accelerated FAD $^{\circ-}$ formation and decay, suggesting that Cys337, Cys523 and possibly Met421

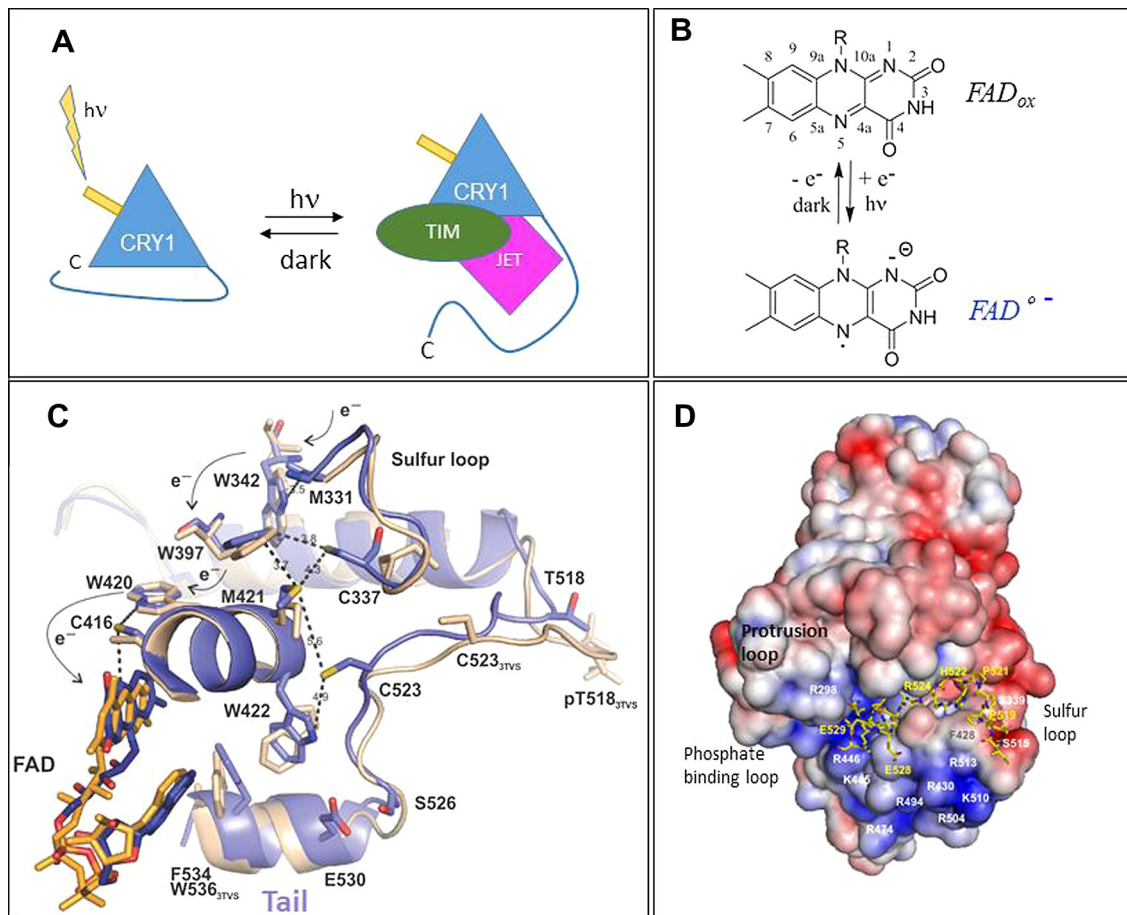


Fig. 4. Structure–function analyses of the phototransduction mechanism of the circadian blue-light photoreceptor *Drosophila* Cryptochrome (dCRY). (A) Light-induced conformational changes of the dCRY tail. Upon blue-light detection by the FAD cofactor, the C-terminal tail of the cryptochrome undergoes a conformational change, which enables binding of dTIM and the E3 ligase JETLAG (JET). That, in turn, will trigger proteasomal degradation of dTIM and CRY, leading to light synchronization of the clock. (B) Photoreaction of FAD in dCRY. FAD in its oxidized form is prevalent in the dark and is converted into the anionic FAD $^{\circ-}$ radical upon blue-light illumination. Adapted from [114]. (C) Crystal structure of full-length dCRY suggests sulfur gated FAD photoreduction. The dCRY structure reported in [95] (dark blue) suggests that electron transfer to oxidized FAD via a cascade of three conserved tryptophanes (Trp342, Trp397, Trp420) is gated by adjacent sulfur rich residues (Cys416 nearby Trp420, Cys337 nearby Trp397, Met331 nearby Trp342). Cys523 in the linker preceding the regulatory tail may sense or affect electron transfer through Met421 located near Trp397. Mutation of the tryptophanes collapsed the photo-reaction in vitro, whereas mutation of Cys416, Cys337 and Cys523 decreased the dark recovery time of oxidized FAD and mutation of Cys337 and Cys523 additionally speeded up photoreduction [95]. Residues Ser526, Glu530 and Phe534 anchor the tail to the dCRY-PHR [95]. Superposition of the full-length dCRY structure reported in [119] (3TVS, beige) reveals a two-amino-acid frameshift throughout the tail, displacement of Cys337 due to altered conformations of tail adjacent loops and different FAD conformations. Due to the 2-residue frame shift in the tail and its preceding linker, critical residues like Cys523, Ser526, Glu530 and Phe534 are displaced in the 3TVS structure, Trp536 (instead of Phe534) is positioned in the location of the photolysis in DNA-repairing photolyases and Thr518 is built as a phosphorylated residue. Figure taken from [95]. (D) Electrostatic surface potential of dCRY suggests binding sites for dTIM and JET. The molecule surface of dCRY can be subdivided into predominantly positively (blue) and negatively (red) charged regions. The tail is depicted as stick presentation in yellow. Light-induced displacement of the tail extends the charged dCRY surface regions, which may facilitate electrostatic interactions with the acidic dTIM or the basic JET molecule. The location of the tail adjacent sulfur-, phosphate binding- and protrusion loops is labeled for orientation. Adapted from [95].

may provide a pathway for crosstalk between the electron transport chain and the regulatory tail [95]. Hence, cysteine reactivity may also play a role in the light-dependent regulation of dCRY, reminiscent of the proposed role of cysteines of mammalian CRY1 in redox sensing (discussed above). Taking cysteine (and potentially methionine) sulfur redox chemistry into account may also help to resolve the controversial discussion about the roles of FAD photoreduction and the classical tryptophan cascade in dCRY function. Notably, in the full-length dCRY structures reported by Czarna et al. [95] and Levy et al. [120] (pdb entries 4K03 and 4GU5), an intermolecular disulfide bond has been observed between Cys296 in the protrusion loops of two adjacent dCRY molecules. However, Berndt et al. have not observed disulfide linked homodimers under gel filtration conditions in solution [114]. While dCRY crystal structures suggest an enhanced redox reactivity of Cys296, a functional role of intermolecular disulfide bond formation in dCRY remains to be identified.

The still open question remains how light-induced conformational changes of the tail regulate dTIM and JET binding to dCRY, which ultimately leads to clock re-synchronization. Analyses of the electrostatic surface potential of dCRY revealed, that the dCRY molecule contains extensive positively and negatively charged surface regions adjacent to the tail, which become even more extended when the tail is removed (Fig. 4D) [95]. Since the dTIM protein is acidic (theoretical pI = 5.17) and the JET protein basic (theoretical pI = 8.54), we postulate that light-induced tail displacement may facilitate dTIM and JET binding to dCRY by removing steric clashes and by generating more extended surfaces for electrostatic interactions.

In vitro and in vivo experiments using structure-based mutations of the discussed cysteines and methionines or of residues within the charged dCRY surface regions, would likely advance our understanding of the phototransduction mechanisms underlying dCRY-dependent light-synchronization of the *Drosophila* circadian clock.

2.8. A final note on Mammalian TIMELESS: a link between the circadian clock, DNA replication and genome stability

In mammals, an orthologue of *Drosophila* TIMELESS was identified by sequence homology. The mammalian TIMELESS (mTIM) orthologue was shown to affect neuronal activity rhythms of the circadian master clock in the SCN, periodicity of peripheral circadian oscillators and DNA damage induced phase advances of circadian clocks [144–146]. The mechanistic role of mTIM within the circadian system could still not be stringently characterized, but may at least partly be correlated with the reported interaction of mTIM with mCRY1 [145]. Notably, mTIM binding to mCRY1 also appears to involve the C-terminal helix of the mCRY1-PHR (α 22) that mediates binding of PER2, FBXL3 and BMAL1 as well as BMAL1/CLOCK transcriptional repression (Fig. 3C) [145]. During the past years, a number of literature reports have described various roles of the mTIM orthologue in DNA replication, in particular in the coordination of helicase and DNA polymerase activities or in checkpoint signaling (reviewed e.g. in [147]). Collective evidence therefore suggests that mammalian TIMELESS has acquired diverse and partly interconnected functions in DNA replication, DNA damage signaling and the circadian clock, presumably defined by its alternate molecular interactions with clock proteins (e.g. mCRY1) or replication fork associated proteins. Furthermore, mTIM is thought to provide a molecular link between the circadian clock and cell cycle regulation.

As the first 3D structural analysis of a TIMELESS protein, the cryo electron microscopy structure and biochemical studies of a complex of mTIM with its interaction partner Tipin (Timeless interacting protein) and the replication protein A (RPA) has

recently been reported [148]. This work has shed light on the 3D architecture of the Timeless-Tipin-RPA complex and the regulation of the recruitment of the mTIM-Tipin complex to the DNA replication fork via RPA conformational changes. Controlled recruitment to different sites and machineries (possibly regulated in time and space) is likely important to allow the multi-tasking mTIM protein to fulfill its diverse functions.

3. Conclusions and perspectives

The discussed 3D structures and structure-based studies of *Drosophila* and mammalian circadian clock proteins have provided important insights into fundamental mechanisms underlying circadian clock function. Crystal structures and protein biochemical analyses of *Drosophila* (d) and mammalian (m) PERIOD proteins as well as of the BMAL1/CLOCK complex have shed light on the regulation of the circadian clock by posttranslational modifications, phosphorylation dependent degradation, cellular translocation and specific interactions of clock proteins. Furthermore, the crystal structures of the mammalian CRY2-FBXL3 and CRY1/2-PER2 complexes illustrate how mPER2 and the E3 ligase FBXL3 bind to the mCRY-PHR in a vastly overlapping manner to control mCRY stability and nuclear translocation and thereby mCRY transcriptional repression activity. Additionally, competitive binding of mCRY to either mPER2 or BMAL1/CLOCK is likely to regulate the transition from early to late repressive BMAL1/CLOCK complexes. The transition from the late repressive to the transcriptionally active state of BMAL1/CLOCK is correlated with acetylation-dependent repressive interactions of the mCRY1 regulatory tail with the BMAL1 TAD as well as binding site competition with the transcriptional activator p300/CBP.

Structure–function analyses of *Drosophila* and mammalian cryptochromes suggest that cysteine chemistry may play a role in the photoreaction and phototransduction of the dCRY circadian photoreceptor and serve as a sensor for the cellular redox state in mCRY1. Completely unexpectedly, the mCRY-PER2 complex structures revealed a jointly coordinated zinc ion in the mCRY-PER2 interface, that stabilizes this complex in mammalian cells structurally and by facilitating reduction of an adjacent intramolecular disulfide bond in mCRY1. Changing cellular redox and metabolic states as well as fluctuating free intracellular zinc concentrations may therefore affect clock function by their impact on the formation and stability of the mCRY-PER2 complex. Consistent with the additional role of mCRY1 in the regulation of glucose homeostasis [149,150], disruption of the zinc interface not only affects clock function but also leads to diabetes symptoms in transgenic mice. A small molecule compound (KL001), that leads to an extended period length, stabilizes mCRY1 by displacing FBXL3 from its interaction site in the FAD cofactor binding pocket and affects gluconeogenesis [20]. The now available crystal structures of mammalian CRYs and their complexes with KL001, FBXL3, FAD and mPER2 will guide the development of improved CRY chemical probes and potentially of novel therapeutic modulators. Such compounds could on one hand affect clock function in a directed manner, and on the other hand be applied in the treatment of metabolic disorders such as type II diabetes. Obviously, molecular chronobiology has gone a long way from the identification of *Drosophila* PERIOD (dPER) as the first clock gene in 1971 to a stage, at which directions for the development of chemical interventions to clock and metabolic disorders are becoming evident. Structural biology has already had and will have a significant impact on understanding the underlying mechanisms and in guiding the rational design of chemical modulators. Furthermore, increasing molecular insights into circadian clock mechanisms and the connections between circadian clocks, physiology and pathogenesis will help

to extend the scope of potential therapeutic applications of compounds that, like KL001, are primarily identified as clock modulating substances.

Wishful thinking? Well, maybe also your jetlag and unease having worked odd hours could be cured by an uncomplicated treatment in future!

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Glossary

Circadian time (CT): By convention, the onset of activity of diurnal organisms defines circadian time zero (CT 0). The onset of activity of nocturnal organisms defines circadian time twelve (CT 12)
 CT 0: beginning of subjective day
 CT 12: beginning of subjective night