To Ub or not to Ub: Regulation of circadian clocks by ubiquitination and deubiquitination

Shashank Srikanta[1,2], Nicolas Cermakian[2,3]*

[1] Integrated Program in Neuroscience, McGill University, Montréal, Québec, H3A 2B4.

[2] Laboratory of Molecular Chronobiology, Douglas Research Centre, Montréal, Québec, H4H 1R3.

[3] Department of Psychiatry, McGill University, Montréal, Québec, H3A 1A1.

* Correspondence can be addressed to Nicolas Cermakian: Douglas Research Centre, McGill University, Montréal, Québec, H4H 1R3, 1-514-761-6131 ext. 4936, nicolas.cermakian@mcgill.ca

ORCID IDs:

Nicolas Cermakian: 0000-0003-4517-9131

Shashank Srikanta: 0000-0002-9437-7241

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List of Abbreviations used in text

BMAL1: Brain and Muscle ARNT-Like 1

CCA1: Circadian and Clock-Associated 1

CLOCK: Circadian Locomotor Output Cycles Kaput

COP1: Constitutive Photomorphogenesis 1

CRY: Cryptochrome

DD: Constant darkness

DUB: Deubiquitinase

FBXL: F-Box And Leucine Rich Repeat Protein

FBXW: F-box and WD repeat domain-containing

FRH: Frequency-interacting RNA helicase

FRQ: Frequency

FWD1: F-box/WD-40 repeat-containing protein 1

GI: Gigantea

JET: Jetlag

KO: Knockout

LD: Light-dark cycle

LHY: Late Elongated Hypocotyl

LL: Constant light

PER: Period

PER2::LUC: PER2 Luciferase fusion protein

PRR: Pseudo Response Regulator

PTM: Post-Translational Modification

REV-ERB: Reverse Erythroblastosis Virus protein

SCF: Skip-Cullin-F-Box Protein Complex

SUMO: Small Ubiquitin-related Modifier

β-TrCP: Beta-Transducin Repeat Containing Protein

TIM: Timeless

USP: Ubiquitin Specific Peptidase

WT: Wild-type

ZTL: Zeitlupe

Abstract

Circadian clocks are internal timing systems that enable organisms to adjust their behavioral and physiological rhythms to the daily changes of their environment. These clocks generate self-sustained oscillations at the cellular, tissue and behavioral level. The rhythm-generating mechanism is based on a gene expression network with a delayed negative feedback loop that causes the transcripts to oscillate with a period of approximately 24 hours. This oscillatory nature of the proteins involved in this network necessitates that they are intrinsically unstable, with a short half-life. Hence, post-translational modifications (PTMs) are important to precisely time the presence, absence and interactions of these genes at appropriate times of the day. Ubiquitination and deubiquitination are counterbalancing PTMs which play a key role in this regulatory process. In this review, we take a comprehensive look at the roles played by the processes of ubiquitination and deubiquitination in the clock machinery of the most commonly studied eukaryotic models of the circadian clock: plants, fungi, fruit flies and mammals. We present the effects exerted by ubiquitinating and deubiquitinating enzymes on the stability, but also the activity, localization and interactions of clock proteins. Overall, these PTMs have key roles in regulating not only the pace of the circadian clock but also their response to external cues and their control of cellular functions.

Introduction to Circadian Rhythms

Circadian clocks are internal timekeepers present in almost all organisms. They generate self-sustained oscillations with a period of ~24 hours (Dibner *et al.* 2010). These circadian rhythms entrain to periodic external signals such as the 24-hour day-night cycle. These self-sustained oscillations affect the organism at the cellular, tissue and behavioral levels and enable organisms to adapt their behavioral and physiological rhythms to the daily changes of their environment (Refinetti 2016).

The rhythm-generating mechanism is based on circadian clock genes, interacting in delayed negative transcriptional-translational feedback loops that cause transcripts and proteins to oscillate with a period of approximately 24 hours. These feedback loops vary both in terms of molecular components and complexity, between different phyla. Despite these differences, the need, across phyla, to maintain a constant period of 24 hours means that there are many commonalities between these different mechanisms. These include PAS domain-containing transcriptional factors that comprise the positive arms of these feedback loops, as well as the post-translational modifications (PTMs) that, together, determine the stability, activity and lifetime of many of the proteins comprised in the clock feedback loops (Stojkovic *et al.* 2014; Loros 2020).

The PTMs in the clock comprise a variety of modifications to the proteins, each of which affect the proteins differently. PTMs such as phosphorylation, acetylation or ubiquitination act on the stability of the proteins, their activity, their interactions, or their intra-cellular localization.

Ubiquitination and Deubiquitination

Circadian clock mechanisms, as well as the rhythmic cellular processes that they control, are based on transcripts and proteins whose abundance varies according to the 24 h cycle. By nature, such rhythmic molecules need to have a short half-life, otherwise they would accumulate and not show a rhythm. This process is mediated by various processes, including post-translational modifications such as ubiquitin tagging of the protein, signalling its degradation. Ubiquitination is therefore one of the important PTMs of the clock machinery, as it tags clock proteins for degradation. A negative feedback loop implies that transcription of genes is stimulated by a set of proteins (the positive arm of the loop) and the protein products of these genes repress the activity of the transcription factors (the negative arm of the loop). Since the components of the positive arm of the loop are mostly regulated at the level of their activity, the reduced protein stability is especially a hallmark of the negative arm of the clock.

Ubiquitin is a 76 amino acid long protein which can be covalently tagged to proteins, post-translationally. Ubiquitin can itself be ubiquitinated, to form straight or branched chain structures. Depending on the length and structure of ubiquitin chains, and on the amino acid being tagged on the target protein, ubiquitination can have distinct functions such as promoting translocation to different cellular compartments, regulating protein-protein interactions or tagging the protein for degradation (Mukhopadhyay and Riezman 2007). For example, ubiquitin chains where ubiquitin moieties are ubiquitinated at Lys48 or Lys11 are generally signals for degradation via the proteasome (Peth *et al.* 2010; Jin *et al.* 2008; Nathan *et al.* 2013).

Three classes of proteins are sequentially involved in the process of ubiquitination: E1, an activating enzyme, E2, a ubiquitin conjugating enzyme, and E3, a ubiquitin ligase. These proteins work in complexes with other proteins, to ubiquitinate the target protein by linking the ubiquitin

at specific residues, generally lysines. There are over 600 E3 ligases, often with specific substrate specificity. As they are involved in the ubiquitin tagging process itself, defects in E3 ligases have been associated with visible changes in the pathways downstream of the protein being tagged (Zheng and Shabek 2017; George *et al.* 2018). There are three main classes of E3 ligases: Really Interesting New Gene (RING), Homologous to E6-AP Carboxyl Terminus (HECT) and Ring Between Ring (RBR) ligases. While RING and HECT domain ligases facilitate the transfer of the ubiquitin moiety from the E2 ligase to the substrate protein, by either acting as a platform (RING) or catalyst (HECT) for this reaction, the RBR ligases contain both RING and HECT domains, hence allowing them to carry out both functions and thus, to ubiquitinate multiple proteins (Zheng and Shabek 2017; George *et al.* 2018). While many different pathways of ubiquitination exist, they are all mediated by members of these three E3 ligase families.

The counterbalance to the process of ubiquitination is achieved by enzymes known as deubiquitinases (DUBs). DUBs work by removing ubiquitin tags from tagged proteins or reducing the length of polyubiquitin tags.. The balance between ubiquitination and deubiquitination impacts physiological protein abundance and activity. This interplay also drives cyclic patterns of protein accumulationin the clock machinery, hence making these PTMs a cornerstone for the rhythmicity of the circadian clock.

In this review, we will focus on the proteins that mediate the processes of ubiquitination and deubiquitination in eukaryotic models most commonly studied for their circadian clock: *Arabidopsis* (plants), *Neurospora* (fungi), *Drosophila* (insects), mice and humans (mammals) (Gallego and Virshup 2007; Stojkovic *et al.* 2014), with a particular focus on the mammalian clock. The role of clock proteins in regulating the ubiquitination/deubiquitination of other proteins will also be addressed.

Ubiquitination and Deubiquitination in the Clock of Plants

The Clock Machinery in Plants

The clock machinery in plants, mainly deciphered through work in the model organism *Arabidopsis thaliana*, is based, as the other eukaryotic clocks, on transcriptional feedback loops. However, this clock mechanism is complex, with many separate, inter-connected loops. The following describes some of the core elements. The expression of the Circadian and Clock-Associated 1 (*CCA1)* and Late Elongated Hypocotyl (*LHY)* genes peak in the morning (Wang and Tobin 1998; Schaffer *et al.* 1998). Their protein products heterodimerize and repress a number of genes via a promoter sequence called the evening element (EE). Given the CCA1/LHY-mediated repression of these genes in the morning, they are expressed at higher levels later in the day or in the evening, when the CCA1/LHY complex is no longer active. This is the case of Time of CAB Expression 1 (TOC 1) (Alabadí *et al.* 2001), an evening transcriptional repressor, which is also called Pseudo Response Regulator 1 (PRR1). TOC1 and other members of the PRR family (PRR5, 7 and 9) are sequentially expressed throughout the day and are involved in a series of repressive events (Farré *et al.* 2005; Nohales and Kay 2016), as shown in Figure 1. The action of these PRR factors leads to a repression of *CCA1* and *LHY* transcription (Farré *et al.* 2005). Of note, CCA1 and LHY also repress their own gene expression (Wang and Tobin 1998; Schaffer *et al.* 1998).

Among other CCA1/LHY targets are the genes encoding Gigantea (GI) as well as components of the Evening Complex (EC), Lux (and its homolog Nox) and Early Flowering 3 and 4 (ELF3 and ELF4). The factors, like TOC1, are active in the evening (Hsu and Harmer 2014). While TOC1 and the other PRRs suppress expression of *CCA1* and *LHY* in the day, GI and the EC will act to

restart the cycle by the activation of *CCA1* and *LHY* (Alabadí *et al.* 2001). Many other factors, including the transcriptional activators of the Light regulated WD (LWD), Reveille (RVE) and Night light–inducible and clock-regulated gene (LNK) families, also add to the mechanism, and provide multiple routes of input from environmental light cues. The description that precedes only scratches the surface of the plant clockwork, in our aim to present its basic players. Readers are referred to other reviews for a more comprehensive description (Nohales and Kay 2016; Hsu and Harmer 2014).

The plant circadian clock regulates most physiological processes, including the cell cycle, the timing of flowering, and abiotic stress responses (Cui *et al.* 2013; Gil *et al.* 2017; Hsu and Harmer 2014). In addition to light, the plant circadian rhythms are also entrainable by other environmental factors such as temperature and nutrient availability (Nohales and Kay 2016; Hsu and Harmer 2014).

Ubiquitin-based PTMs in plants

The activity and stability of the various proteins involved in the plant circadian clock is tightly regulated by PTMs, including ubiquitination. The circadian photoreceptor and F-box protein Zeitlupe (ZTL) associates with Skip and Cullin to form a Skip-Cullin-F-Box (SCF) E3 ligase complex (Kim *et al.* 2007). This SCF complex ubiquitinates and targets TOC1 and PRR5 for proteasomal degradation (Más *et al.* 2003; Kiba *et al.* 2007). ZTL was also shown to contribute to the thermostability of the circadian clock via its ligase function. ZTL KO resulted in the plant having lower thermotolerance as well as an increase in protein accumulation (Gil *et al.* 2017). The degradation of ZTL itself, proceeds via a ubiquitination-dependent mechanism (Kim *et al.* 2003). Interestingly, another core clock protein, GI, associates with ZTL in a blue light dependent manner,

and the two proteins reciprocally stabilize each other. Their subsequent dissociation in darkness provides a precise input of photoperiod (Kim *et al.* 2007).

Flavin-Binding, Kelch Repeat, F-Box1 (FKF1) and LOV Kelch Protein 2 (LKP2) are the other members of the ZTL family of F-box proteins, involved in the regulation of the plant circadian clock (Nelson *et al.* 2000; Yasuhara *et al.* 2004; Han *et al.* 2004). Moreover, in triple mutants of the ZTL family, a significant period lengthening phenotype and a reduction of *LHY* gene expression were observed, thus underlining the importance of these proteins in the clock machinery (Lee *et al.* 2018; Baudry *et al.* 2010).

Ubiquitin Specific Peptidases or USPs (also known as Ubiquitin Binding Peptidases or UBPs) are the largest class of DUBs in plants. USP12 and USP13 (UBP12 and UBP13) are special in that they are regulated by the circadian clock and they also regulate LHY, CCA1, ZTL, GI and TOC1, all core clock components (Lee *et al.* 2019). Moreover, USP12 and 13 also regulate downstream processes such as the daily timing of flowering (Cui *et al.* 2013; Park *et al.* 2019; Lee *et al.* 2019).

Using over-expression and KO-based studies, USP15 and USP26 were implicated in early flowering phenotypes, by regulating non-circadian genes downstream of the circadian clock (Liu *et al.* 2008; Schmitz *et al.* 2009). Recent studies have shown that Ubiquitin Carboxyl Hydrolases 1, 2 and 3 (UCH 1/2/3), another family of DUBs, act in conjunction to maintain circadian periodicity in varying temperature conditions, although the targets of these UCHs remain unknown (Hayama *et al.* 2019).

Light input pathways also rely on ubiquitination. Besides the ZTL family members which are photoreceptors (Kim *et al.* 2007), another pair of photoreceptors, Cryptochrome 1 and 2 (CRY 1 and 2) are involved in the light-mediated regulation of clock proteins via ubiquitination. In response to light, these CRYs bind and activate Constitutive Photomorphogenesis 1 (COP1), a

RING domain E3 ligase (Wang *et al.* 2018; Yang *et al.* 2001). COP1, in conjunction with ELF3 (a member of the EC, which acts as a substrate adaptor here), destabilizes GI and tags it for degradation (Yu *et al.* 2008).

In conclusion, the processes of ubiquitination and deubiquitination intricately control and are controlled by the circadian clock machinery, hence making them key PTMs involved in the maintenance of a multitude of physiological rhythms in plants. The importance of ubiquitination in the plant clock is probably much greater than described above: a recent screen of *Arabidopsis* strains expressing dominant negative forms of ubiquitin ligases has identified dozens of new ligases involved in regulating circadian rhythms, having from minor to more extensive effects on period or phase of the rhythms (Feke *et al.* 2019).

Ubiquitination and Deubiquitination in the Clock of *Neurospora*

The clock in *Neurospora*

The fungus *Neurospora crassa* was one of the earliest model organisms studied for their circadian clock mechanisms (Dunlap and Feldman 1988). Like in other organisms, the clock in *Neurospora* is based on an auto-regulatory feedback loop consisting primarily of four proteins. The transcription factors White Collar 1 and 2 (WC1 and WC2) form the White Collar Complex (WCC) (Crosthwaite *et al.* 1997), which induces transcription of *frequency* (*frq)* (Froehlich *et al.* 2002) (Figure 2). FRQ protein forms homodimers, which in turn associate with the Frequency-interacting RNA helicase (FRH) protein to form the FRQ/FRH complex (FFC) (Cheng *et al.* 2005). The FFC binds to the WCC complex and prevents its binding to the *frq* locus (Froehlich *et al.* 2003; Guo *et al.* 2010). Later in the cycle, FRQ needs to be degraded, and a new cycle can start. FRQ dimers also regulate WC1 protein at the post-translational level (Lee *et al.* 2000; Cheng *et*

al. 2001). Finally, FRH being an RNA-binding protein, it can regulate *frq* mRNA's stability and poly A tail length (Guo *et al.* 2009). Overall, the period of the *Neurospora* clock feedback loop is set by the synthesis and turnover of FRQ, which, in turn, is controlled by its interactions with itself and with the other clock proteins.

Ubiquitin-based PTMs in *Neurospora*

FRQ stability is tightly regulated via its ubiquitination. F-box/WD-40 repeat-containing protein 1 (FWD1) is required for this PTM (He and Liu 2005; He *et al.* 2003; Guo *et al.* 2010). Nonsense point mutations in the F-box domain of FWD1 resulted in higher levels of FRQ, which was also hyperphosphorylated. The rhythmicity of FRQ was lost, due to a lack of ubiquitination. Complete deletion of the F-box domain of FWD1 also led to the same phenotype. Interestingly, in both cases, in the absence of ubiquitination activity, hyperphosphorylated FRQ was bound to FWD1 for longer than in WT cells, showing the requirement of ubiquitination for the dissociation of FRQ from FWD1. Further, altered conidiation patterns were also observed in these mutants: these patterns were not consistent, between individual samples, but in many cases, initially, low amplitude rhythms of conidiation were observed, which evolved into arrhythmicity over time (He *et al.* 2003).The above studies show the essential role of FWD1 in recruiting FRQ to the SCF complex for FRQ degradation.

The COP9 signalosome (CSN) is an 8-subunit complex that can remove NEDD8, a ubiquitin-like molecule, from any tagged protein, by a process called deneddylation. The Cullin subunit of the SCF^{FWD1} complex is tagged with a NEDD8 molecule while it is part of the SCF complex. This tag prevents its association with Cullin Associated And Neddylation Dissociated 1 (CAND1), a protein for which Cullin has greater affinity than for the Skip-F-box complex. Hence,

deneddylation of Cullin by the CSN causes disassembly of the SCF complex, which inhibits ubiquitination of FRQ (Cope and Deshaies 2003; Wei and Deng 2003). Interestingly, CSN also seems to be essential for the ubiquitination activity of SCF. Studies showed that the CSN has a twofold role in this process. First, it is able to stabilize the SCF complex, thus promoting ubiquitination. Second, by deneddylating Cullin and allowing the SCF complex to dissociate, it guards against the autoubiquitination activity of SCF complexes which remain in bound conformation for too long. Thus, the CSN complex is essential to the timely ubiquitination of FRQ by SCF^{FWD1} complex (Wolf *et al.* 2003; Lyapina *et al.* 2001). CSN mutants display FRQ accumulation and a loss of rhythmicity, which further underscores the importance of the CSN in this process (He *et al.* 2005).

Conidial separation 1 (CSP1) is a transcription factor which is clock-controlled and hence, rhythmically expressed (Sancar *et al.* 2011). WCC directly activates the expression of the *CSP1* gene at the beginning of the subjective day. Thereafter, CSP1 represses the transcription of many genes which are generally transcribed in the evening. The degradation of CSP1 later in the day allows the transcription of these genes. Thus, CSP1 degradation is important to the regulation of evening-specific genes in *Neurospora*. This degradation of CSP1 is regulated by the E3 ubiquitin ligase UBR1. In single copy deletion mutants of *ubr1*, CSP1 accumulates in its hyperphosphorylated form, thus showing the necessity of UBR1 to the degradation of CSP1 (Sancar *et al.* 2011).

Ubiquitination and Deubiquitination in the Clock of *Drosophila*

The Clock in *Drosophila*

The circadian clock of the fruit fly *Drosophila melanogaster* is one of the most widely studied clock systems. Many components of the animal clock machinery were initially isolated via loss-of-function genetic screens in *Drosophila*. In the *Drosophila* clock, Cycle (CYC) associates with Clock (CLK) and they induce the transcription of *period* (*per)* and *timeless* (*tim)* genes (Figure 3). PER and TIM proteins then translocate into the nucleus and inhibit the activity of the CLK/CYC complex, and thus, their own expression (Williams and Sehgal 2001). The clock also has a secondary feedback loop, which is made up of Vrille (VRI) and PAR Domain Protein 1ε (PDP1ε), which repress and activate, respectively, the expression of *clk* (Helfrich-Förster *et al.* 2020).

The *Drosophila* master clock is localized within the head of the flies, while peripheral organs also show autonomous oscillations, under the control of the master oscillator (Helfrich-Förster 2004). This master clock is localized to discrete groups of neurons termed as the lateral neurons (LN) and dorsal neurons (DN). A hallmark of these cells is the strong *per* and *tim* oscillations, and for some subsets of these neuronal populations, the presence of the neuropeptide PDF (pigment dispersing factor) (Helfrich-Forster 1995; Schubert *et al.* 2018). These LN cells drive peripheral clocks via electrical or humoral signals (Helfrich-Förster 2004).

Ubiquitin-based PTMs in *Drosophila*

Supernumerary limbs or SLIMB (SLMB) is a well characterized F-box protein in *Drosophila* (Jiang and Struhl 1998). SLMB was previously known to be an E3 ligase, but it was only in 2002 that it was shown to have a role in circadian rhythms (Grima *et al.* 2002; Ko *et al.* 2002). SLMB ubiquitinates PER and hence facilitates its degradation (Chiu *et al.* 2008). Prior to ubiquitination,

PER has to be phosphorylated by the kinase Doubletime (DBT). SLMB was also shown to ubiquitinate TIM for degradation. Flies lacking *slmb* showed an accumulation of phosphorylated PER and TIM (Grima *et al.* 2002). Constitutive expression of *slmb* led to arrhythmicity in DD, but not in LD. Further, expression of a dominant form of SLMB lacking the F-box domain also led to the flies showing long periods or arrhythmicity in DD (Ko *et al.* 2002).

The *Drosophila* system has a type of cryptochrome known as type 1 Cryptochrome or CRY. This class of CRY proteins (found exclusively in invertebrates) are photoreceptors and contribute to light reception signaling (Emery et al. 1998; Stanewsky et al. 1998). CRY is activated in response to light stimulation, and then binds TIM and PER (Emery *et al.* 1998; Emery *et al.* 2000; Rosato *et al.* 2001) and facilitates their translocation into the nucleus (Rosato *et al.* 2001). In the presence of E3 ligase Jetlag (JET), CRY and JET complex with TIM, to prevent nuclear localization and facilitate ubiquitination of TIM (Lin *et al.* 2001; Koh *et al.* 2006). The subsequent degradation of TIM leads to clock resetting (Naidoo *et al.* 1999; Lin *et al.* 2001; Koh *et al.* 2006). *Cry* KO flies are resistant to light-induced phase shifting, as well as free-running in constant light conditions (LL) (Stanewsky et al. 1998; Tataroglu and Emery 2015; Krishnan et al. 2001; Damulewicz and Mazzotta 2020). Mutants of *jet* on the other hand, show no alterations in phenotypes in either LL or DD. In LD, however, these flies took significantly longer to entrain to phase shifts (Koh *et al.* 2006). Put together with the phenotype of SLMB mutants being arrhythmic in DD, we can hypothesize that JET and SLMB complement each other's function in the regulation of TIM.

Circadian Trip (CTRIP), a HECT domain family E3 ubiquitin ligase, was identified to increase the ubiquitination of CLK, in *Drosophila*. It is prominently expressed in the PDF cells and a loss-of-function mutant has an increased circadian period. There is also evidence suggesting that

CTRIP plays a role in the degradation of the PER/TIM complex. Hence, CTRIP is an E3 ubiquitin ligase involved in both the positive and negative arms of the *Drosophila* circadian feedback loop (Lamaze *et al.* 2011).

Nipped-A is a deubiquitinase which has a roundabout effect on circadian rhythms in *Drosophila* (Bu *et al.* 2020). Nipped-A deubiquitinates histone H2B, at the *tim* and *pdp1ε* loci. Deubiquitination of these ubiquitinated loci leads to an increase in the transcription of these genes. The effect on the *tim* locus seems to drive the long-period phenotype of the knockdown of *Nipped-A*. Indeed, *clk* mRNA levels are unchanged in the knocked down flies, in spite of a reduction of PDP1ε. Mutations of the human homolog, TRRAP, are associated with schizophrenia (Bu *et al.* 2020), but its involvement in the circadian system has not been studied yet.

USP8 is a clock-controlled deubiquitinase: its transcription is activated by CLK/CYC complex, with a phase similar to that of *tim* and *per*. USP8, in turn, deubiquitinates CLK early at night. USP8 knockdown led to an increase in the free-running period of the flies. Almost 50% of flies were arrhythmic in the absence of USP8. Mutants with a mutation in the catalytic domain showed a longer period (Luo *et al.* 2012).

Ubiquitination and Deubiquitination in the Clock of Mammals

The Clock in Mammals

In the mammalian clock, the transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK) (Gekakis *et al.* 1998) and Brain and Muscle ARNT-Like 1 (BMAL1) (Takahata *et al.* 1998) together bind E-box elements upstream of the *Period* (*Per1, 2, 3)* and *Cryptochrome (Cry1, 2)* genes, activating their transcription (Figure 4). After translation, the PER (Vitaterna *et al.* 1999; Akiyama *et al.* 1999) and CRY (Miyamoto and Sancar 1998; Kume *et al.* 1999; Vitaterna *et al.*

1999; Van Der Horst *et al.* 1999) proteins translocate into the nucleus, interact with each other and suppress the activity of the CLOCK/BMAL1 complex, reducing the transcription of *Per* and *Cry* and hence completing one 24-hour cycle of the circadian clock. This main feedback loop is flanked by subsidiary feedback loops such as those of Reverse Erythroblastosis Virus proteins (REV-ERBs (Preitner *et al.* 2002; Guillaumond *et al.* 2005) and RAR-related orphan receptors (RORs) (Sato *et al.* 2004; Guillaumond *et al.* 2005). The genes encoding these proteins are transcribed by the CLOCK/BMAL1 complex. While the REV-ERBs inhibit *Bmal1* expression, the RORs activate it. All these proteins also have targets outside the clock machinery. Due to the circadian patterns of expression and activity of clock transcription factors, their downstream targets can also be expressed in a circadian fashion. Such genes are called clock-controlled genes (Zhang *et al.* 2014). These clock-controlled genes have a circadian pattern of expression and have short half-lives, but they are not a part of the circadian clock.

Most cell types in the body express clock genes and therefore have the capacity to generate autonomous circadian rhythms. A master clock, present in a brain region called the suprachiasmatic nucleus (SCN), synchronizes the other body clocks via various neuronal, humoral and systemic cues (Dibner *et al.* 2010; Hastings *et al.* 2019).

Ubiquitin-based PTMs in mammals

As in other eukaryotes, mammalian clock proteins undergo ubiquitination, which is key to the functioning of the molecular clockwork. In the upcoming sections, we will explore the ubiquitination and deubiquitination of each of the core clock proteins, as well as the roles of these clock proteins themselves in regulating the ubiquitination/deubiquitination of other proteins.

Cryptochrome (CRY) proteins

Ubiquitination

The first known mediator of degradation of CRY proteins was F-Box And Leucine Rich Repeat Protein 3 (FBXL3). This F-box protein associates with Skip and Cullin to form the SCF^{FBXL3} E3 ubiquitin ligase complex, which ubiquitinates CRY proteins and targets them for degradation. Loss-of-function mutations of *Fbxl3* revealed an increased half-life of CRYs as well as a longer free-running period in these mutant mice (Busino *et al.* 2007; Siepka *et al.* 2007; Godinho *et al.* 2007). CRY proteins have two major functional domains: while one is necessary for CRY/PER interactions, the other (conserved) one can bind FADH (required for the photoreception activities of cryptochromes in other organisms such as plants and invertebrates.). While there is no evidence of CRY-mediated photoreception in mammals, the FAD-binding domain acts as the primary docking site for FBXL3. After docking, SCF^{FBXL3} acts by binding and inactivating both the FAD- and PER-binding domains of CRY2. This interaction was reduced in the presence of PER2 (Xing *et al.* 2013). Accordingly, KL001, a small molecule identified in a cell-based circadian screen, was shown to bind the FAD-binding pocket of CRY proteins, and by doing so, to inhibit the binding of FBXL3 and to stabilize CRYs (Hirota *et al.* 2012).

FBXL21 is a paralog of FBXL3, which is also involved in the degradation of CRY proteins. *Fbxl21* gene displays a circadian expression (Dardente *et al.* 2008). Interestingly though, despite the similarity of the two ubiquitin ligases, mice that lack (Hirano *et al.* 2013) or contain a mutated form (Yoo *et al.* 2013) of FBXL21 display either no change in free-running period or a small reduction, in contrast to the very long period of mice lacking FBXL3 function (Siepka *et al.* 2007; Godinho *et al.* 2007), while the double KO or mutant mice show intermediate phenotypes, rather than an additive effect (Yoo *et al.* 2013; Hirano *et al.* 2013). The explanation for this phenotype is

that, at the cellular level, FBXL21 and FBXL3 are spatially separated and have distinct — rather than redundant — roles in the mammalian clock. CRY protein levels generally peak in the night (Yoo *et al.* 2013). FBXL21 is involved in the degradation of CRYs in the cytoplasm, where it forms an SCF complex that slowly degrades CRYs and prevents them from peaking too early (Hirano *et al.* 2013). In the early night, FBXL3 begins to ubiquitinate CRYs in the nucleus for degradation. FBXL21 is present in the nucleus as well and has a higher affinity for CRYs than FBXL3, but a lower ubiquitinating activity. Thus, FBXL21 reduces the association of CRYs to FBXL3, which prevents it from targeting CRYs for degradation too quickly. This antagonistic relationship between FBXL3 and FBXL21 is essential to ensuring an adequate build up and subsequent decrease of CRY protein levels and thus setting the period of the clock. This model is consistent with the effect of mutations on the period in mice (Yoo *et al.* 2013; Hirano *et al.* 2013).

F-box and WD repeat domain-containing 7 (FBXW7) is another F-box protein that might be regulating CRY proteins. Using colorectal cancer cells, a link was established between FBXW7 and CRY2. FBXW7 bound CRY2 phosphorylated at Thr300, and the overexpression of FBXW7 led to a decrease in CRY2 levels and half-life (Fang *et al.* 2015). Of note, another study described later in this review, and focusing on FBXW7 and REV-ERBα mentioned (but did not show data) that this ubiquitin ligase does not bind CRYs (Zhao *et al.* 2016). This discrepancy, and the in vivo relevance of the FBXW7-CRY interaction, remains to be clarified. Further, it would be of interest to understand how the activity of FBXW7 ties into the FBXL3-FBXL21-CRY regulatory pathways.

Another ubiquitin ligase implicated in the degradation of CRYs is a protein complex consisting of Cullin 4-Damaged DNA-binding protein 1 (CUL4-DDB1) and CDT10-dependent transcript 2 (CDT2, also named DCAF2). The ubiquitin ligase activity on CRY1 was ascribed to

CUL4-DDB1 whereas CDT2 is important for the binding of the complex to CRY1. Ubiquitination assays and co-IP experiments showed that CUL4-DDB1 ubiquitinates and destabilizes CRY1, and knockdown of DDB1 stabilizes it. Mutating Lys585 of CRY1 (the residue being ubiquitinated) to an alanine causes it to become insensitive to the ligase complex and show elevated protein levels. In cells with either a mutant DDB1 or ligase-insensitive CRY1, the amplitude of bioluminescence circadian rhythms was increased, although the period remained unchanged. Thus, CUL4-DDB1-CDT2 is a novel ubiquitin ligase complex of CRY proteins in the clock machinery (Tong *et al.* 2015).

Deubiquitination

USP2 is a rhythmically expressed DUB that was shown to be involved in the deubiquitination of CRY1 (Tong *et al.* 2012). USP2 is a particularly interesting protein, with respect to the clock. On one hand, the gene is widely expressed in the body and presents rhythmic expression patterns in all the tissues that express it (one of the only genes to have this property) (Yan *et al.* 2008; Zhang *et al.* 2014). On the other hand, USP2 is intricately involved with the clock and contributes to the deubiquitination of multiple clock proteins, which will be addressed further in the text. These properties make USP2 an important deubiquitinase in the circadian system. Specifically, with respect to CRY proteins, USP2 is involved in regulating the stability of the CRY1 protein. Serum stimulation of U2OS cells led to the induction of USP2, as well as the stabilization of CRY1. Knocking down *Usp2,* in cultured cells or in the mouse liver, resulted in decreased CRY1 protein levels and increased ubiquitination patterns. Thus, USP2 modulates the half-life of CRY1 (Tong *et al.* 2012).

More recently, USP7 (also known as Herpes-activated USP or HAUSP, due to an alternate mode of activation of this protein, in the presence of Herpes viral protein) was shown to deubiquitinate both CRY proteins. ShRNA-mediated knockdown of *Usp7* in fibroblasts showed an increase in the ubiquitination of CRYs and hence, a decrease in CRY protein levels (Papp *et al.* 2015; Hirano *et al.* 2016). As expected, the knockdown of *Usp7* resulted in a reduction of circadian period (also seen in *Fbxl3* overexpression mutants). More interestingly, *Usp7* overexpression in *Fbxl3* knockdown cell lines still resulted in greater stabilization of CRY proteins than in *Fbxl3* knockdown controls, hence pointing towards the role of USP7 as a deubiquitinase that is not specific to ubiquitination by a specific ligase (Hirano *et al.* 2016). A recent study involving the Melanoma Antigen L2 protein (MAGEL2) protein has further refined the understanding of the USP7-CRY relationship. In this paper, it was shown that MAGEL2, a protein known to interact with ubiquitin ligases and DUBs, interacts with USP7 and prevents its binding to CRY proteins. Furthermore, *MAGEL2* overexpression led to a reduction in CRY levels, showing that USP7 is required for the deubiquitination and stability of CRY proteins (Carias *et al.* 2020). The circadian expression of *MAGEL2* suggests that the phased activity of USP7 might be regulated by the presence of MAGEL2.

The role of CRYs in ubiquitination

Recent studies have shown that many clock proteins are also involved in modulating the ubiquitination of other proteins, by binding to ligases as cofactors. This function of CRYs is especially well studied. The SCF^{FBXL3} complex has two separate binding sites for CRYs: while binding to one of them blocks the active site of CRY proteins and causes their polyubiquitination, binding to the other site allows CRYs to act as cofactors to the SCF^{FBXL3} complex and promotes

the ubiquitination of other targets of the SCF^{FBXL3} complex. The SCF^{FBXL3} complex recruits the CRYs to ubiquitinate proteins involved in the cell cycle, such as Tousled-like Kinase 2 (TLK2) (Correia *et al.* 2019; Huber *et al.* 2016).

A major player in regulating the cell cycle is c-Myc. This protein is a transcription factor and activates genes involved in cell growth and proliferation. Interestingly, CRY2 (but not CRY1) is part of the FBXL3 E3 ligase complex that was recently shown to stimulate the degradation of c-Myc. In accordance with this finding, a loss of CRY2 not only stabilized c-Myc, but also resulted in increased proliferation of the CRY2-deficient cells (Huber *et al.* 2016).

Another important protein family involved in the cell cycle is the E2F family of transcription factors (including activators and repressors of the cell cycle). These proteins are key to the rate of progression of the cell cycle (especially the G-to-S phase transition) and hence, their expression and degradation pathways need to be tightly regulated. The $SCF^{FBXL3+CRY2}$ complex has been implicated in this process of regulation of the repressive members of the E2F family. In the absence of CRY2, the cell cycle progresses more rapidly, due to reduced ubiquitination of E2Fs (Chan *et al.* 2020).

Within the context of functioning of the core clock itself, CRY proteins are thought to modulate the ubiquitination and stability of PER proteins. PER2 levels are strongly reduced in *Cry* double KO mouse tissues (Shearman *et al.* 2000). In *Cry* double KO cells, in the presence of a proteasome inhibitor, ubiquitinated PER2 levels were higher. CRYs bind to PER2, prevent its export from the nucleus and thus, its ubiquitination (Yagita *et al.* 2002).

Period (PER) proteins

Ubiquitination

PER proteins have the shortest half-lives among clock proteins (D'Alessandro *et al.* 2015). Hence, it is of paramount importance that the ubiquitination and deubiquitination be tightly regulated to maintain the 24-hour rhythmicity. Beta-Transducin Repeat Containing Protein (β-TrCP1 or FBXW1) and β-TrCP2 (FBXW11) are homologs of *Drosophila* SLIMB. In mammalian cells, the β-TrCP proteins bind to PER proteins and stimulate their degradation (Reischl *et al.* 2007; Ohsaki *et al.* 2008). Knockdown of *β-TrCP* expression dampens circadian rhythms (Reischl *et al.* 2007; Ohsaki *et al.* 2008), due to a reduction in the transcription of *Per* and *Cry* genes, possibly as a result of accumulation of PERs and hence, increased inactivation of the CLOCK/BMAL1 complex. As a result, the circadian period was lengthened in cells (Reischl *et al.* 2007). Interestingly though, the free-running period of locomotor activity rhythms was not altered in *β-TrCP1* KO mice compared to controls, possibly due to redundancy with *β-TrCP2* (Ohsaki *et al.* 2008).

Addressing such a redundancy of β-TrCPs for their action on PER proteins in vivo was made difficult by the essential role of β-TrCP2 during development, such that KO of this gene is embryonically lethal. To circumvent this, D'Allessandro et al. generated inducible *β-TrCP2* KO mice. Inducing KO of this gene in adult mice allowed to see a reduced amplitude and an increased period of locomotor activity rhythms, unlike what was seen in cells (D'Alessandro *et al.* 2017). Notably, crossing these *β-TrCP2* KOs with *β-TrCP1* KO mice led to a strong worsening of the circadian rhythms (D'Alessandro *et al.* 2017), supporting the redundancy of these ubiquitin ligases, and their crucial role in the clock mechanism.

The recruitment of β-TrCP is conditional on specific phosphorylation of PER proteins. Casein Kinase 1ε (CK1ε) is the kinase that is involved in this process. CK1ε interacts directly with PER2 and when the activity of CK1ε is blocked, the circadian period is observed to lengthen, and degradation of PER proteins also reduces (Eide *et al.* 2005). Thus, CK1ε has a central role in regulation of PER stability. Interestingly though, despite the effect of β-TrCPs on PER protein stability, PER ubiquitination still occurs in *β-TrCP* double KO cells, indicating that other ligases can act on PER proteins. Thus, it was suggested that β-TrCPs were acting specifically on the pool of hyperphosphorylated PER proteins (D'Alessandro *et al.* 2017).

Ubiquitin protein ligase E3 component N-recognin 4 (UBR4) is an E3 ligase of the N-end rule pathway with a potential role in PER2 ubiquitination and degradation. UBR4 protein peaks with a phase that coincides with the beginning of PER2 degradation in the SCN (early night) (Ling *et al.* 2014). More research will be needed to define the implication of this ubiquitin ligase in clock function.

Deubiquitination

Two DUBs were shown to counteract the effect PER protein ubiquitin ligases. One of them is USP2, which was discussed above as a CRY1 deubiquitinase. Mice KO for USP2 show a slightly longer free-running period than WT mice. Interestingly, although USP2 associates with PER1 and promotes its deubiquitination, it seems to have no effect on the stability of PER proteins (Yang *et al.* 2012). Instead USP2 regulates the intracellular localization of PER1, and *Usp2* KO cells display reduced PER1 levels in the nucleus (Yang *et al.* 2014). A possible role of USP2 in regulating the response of the clock to light was also investigated. A notable impact of *Usp2* KO was found: the response of *Usp2* KOs to light signals causing phase delays (early night light pulse, or delay in the

light-dark cycle) was enhanced, whereas the response to stimuli causing phase advances was largely reduced (Yang *et al.* 2012).

USP14 is another, recently discovered, DUB regulating PER protein levels. USP14 reduced the levels of polyubiquitinated PER1 (D'Alessandro *et al.* 2017). In the presence of a dominant negative form of USP14 in cells, PER2 was destabilized and the period of a bioluminescent reporter (a proxy for clock function) was shortened (D'Alessandro *et al.* 2017).

The role of PER proteins in ubiquitination

P53 is a cell cycle checkpoint transcription factor. The activation of p53 results in G2/M transition arrest in the cell cycle and promotes cell apoptosis (Taylor and Stark 2001). This protein is generally activated in response to DNA damage in the cell. PER2 was found to bind and inactivate p53 (Gotoh *et al.* 2014). Another function of PER2 is to prevent the degradation of p53 by blocking its ubiquitination by the murine double minute 2 (MDM2) E3 ligase. At the end of the G2 phase, PER2 dissociates from p53, which allows MDM2 to ubiquitinate it for degradation. In conditions of genotoxic stress, it was shown that PER2 dissociates from p53, hence allowing it to transcribe proapoptotic genes (Gotoh *et al.* 2015).

In the section about CRY proteins, we saw that CRYs protect the PERs from degradation. The PER proteins return the favor. It was observed that CRY1/2 levels went down in *Per1/2* double KO mice (Bae *et al.* 2001). Furthermore, CRY ubiquitination decreased in a dose-dependent manner in the presence of PER2 (Yagita *et al.* 2002). The mechanism seems to be based on the binding of PER2 to the cofactor pocket of CRY proteins, hence preventing their ubiquitin ligases from binding to them (Xing *et al.* 2013). Also, the export of PER and CRY proteins out of the

nucleus is reduced when they are in complex with each other (Yagita *et al.* 2002). Thus, in complex, PER and CRY proteins inhibit each other's ubiquitination.

BMAL1

Ubiquitination

The CLOCK/BMAL1 complex, as a central player in the molecular clock, requires a precise and timely regulation of its activity. This involves a number of PTMs of these proteins, including well-orchestrated ubiquitination events.

A serendipitous experiment led to the discovery of a role for Ubiquitin protein ligase E3A (UBE3A) in the ubiquitin tagging of BMAL1 for degradation (Gossan *et al.* 2014). In experiments initially aimed at immortalizing mouse embryonic fibroblasts (MEFs), harvested from PER2::LUC mice, using oncogenes E6/E7, Gossan and colleagues observed severely dampened bioluminescence rhythms, and reduced BMAL1 expression levels and stability. They suggested that this could be due to ubiquitination of BMAL1 by UBE3A, an E6-associated ubiquitin ligase. Further study using a mutant UBE3A lacking ligase activity indeed showed an accumulation of BMAL1. Bioluminescence assays in knockdown and overexpression models of *Ube3a* showed an increase and decrease in period, respectively. Ube3a knockdown experiments in *Drosophila* further indicated a role of this ubiquitin ligase in regulating circadian rhythms (Gossan *et al.* 2014).

BMAL1 is also ubiquitinated by Ubiquitin conjugating enzyme E2 O (UBE2O). This is, interestingly, an E2 ligase, which is actually an E2/E3 hybrid (also referred to as E3-independent). Experiments in U2OS cells have shown that UBE2O is able to ubiquitinate and hence promote degradation of BMAL1. Knocking down *Ube2o* resulted in an increase in the amplitude of the

bioluminescence rhythms being recorded from these cells, while also stabilizing BMAL1(Chen *et al.* 2018b).

TNF receptor associated factor 2 (TRAF2) is another recently identified ubiquitin ligase that seems to modulate the ubiquitination of BMAL1 (Chen *et al.* 2018a). Overexpression of TRAF2 in HEK293 cells resulted in a 2.5-fold reduction of BMAL1 half-life. Further, TRAF2 and BMAL1 physically interacted to mediate the E3 ligase activity of TRAF2. This interaction led to an increase in the rate of BMAL1 degradation, indicating that TRAF2 ubiquitinates and hence, promotes degradation of BMAL1 (Chen *et al.* 2018a).

The E3 ligase STIP1 homology and U-box–containing protein 1 (STUB1) ubiquitinates BMAL1 in the context of cellular ageing (Ullah *et al.* 2020). STUB1 was identified in a screen for BMAL1 partners. The overexpression of STUB1 downregulated BMAL1 protein levels, while they increased in *Stub1* knocked-down HEK293 cells. Further, STUB1 promoted polyubiquitination of BMAL1. Although the impact of this interaction on circadian rhythms was not investigated, this process was found to take place during conditions of oxidative stress (Ullah *et al.* 2020). Whether this interaction takes place even in homeostatic conditions and what its effects are, still needs to be studied.

Small Ubiquitin-related Modifier (SUMO) is a protein related to ubiquitin. This is also a PTM and follows a pathway of attachment similar to the ubiquitin ligation pathway (Gill 2004). BMAL1 was found to be SUMOylated at its Lys259 in the presence of the CLOCK. This SUMOylation event occurred in a rhythmic fashion in the mouse liver, and peaked in the late subjective day, i.e. the time of highest CLOCK/BMAL1 transcriptional activity. A form of BMAL1 mutated at Lys259 was more stable, and cells with this mutant BMAL1 showed altered circadian gene expression (Cardone *et al.* 2005). Further studies revealed that SUMO 2/3 are essential to the

activation of the CLOCK/BMAL1 complex, as well as to the ubiquitination and subsequent degradation of BMAL1. Blocking the proteasomal degradation pathway led to accumulation of BMAL1 molecules that were tagged not only with ubiquitin, but also with SUMO 2/3 (Lee *et al.* 2008). The activation of CLOCK/BMAL1 complex in response to SUMOylation was elucidated in a later study, where SUMOylation was shown to be essential to the binding of the co-activator CREB binding protein (CBP) (Lee *et al.* 2015).

Deubiquitination

The deubiquitination of BMAL1 involves at least two DUBs, USP2 and USP9X. In 2008, Lee and colleagues were the first to find that BMAL1 was being deubiquitinated by a protein known as UBP41, which was later shown to be the 45 kDa isoform of the USP2 protein (Lee *et al.* 2008; Gousseva and Baker 2003). This result was confirmed using biochemical and mouse behavioral studies. BMAL1 protein levels were reduced in the SCN of *Usp2* KO mice, especially in the late day and early night, a time when CLOCK/BMAL1 has its highest activity. Accordingly, PER1 levels are also reduced around those times (Scoma *et al.* 2011). Further, the KO mice showed only minor phenotypes in their light responses, and no difference in their free-running period, compared to WT mice (Scoma *et al.* 2011). This is surprising, considering the effects on BMAL1 stability and abundance, and given the different results in the other *Usp2* KO line described above (PER section), which have an altered free-running period and a clear light response phenotype (Scoma *et al.* 2011; Yang *et al.* 2012).

More recently, cell culture studies identified USP9X (also known as UCH FAF-X) as another potential BMAL1 DUB (Zhang *et al.* 2018). Overexpression of USP9X in HEK293 cells showed a marked increase in the stability and abundance of BMAL1, with the complementary

bioluminescence studies showing higher amplitude circadian oscillations. Knocking down *Usp9X* via siRNA supported the above results, by presenting a marked decrease in BMAL1 levels (Zhang *et al.* 2018). Based on the slight phenotypes detailed above, it is possible that USP2 and USP9X, and perhaps other DUBs, might act on BMAL1 in a partly redundant manner.

Finally, Protein Kinase C γ (PKCγ) was shown to inhibit ubiquitination of BMAL1 in HEK293 cells and increase its stability. Interestingly, it is also able to unconventionally promote deubiquitination of BMAL1: PKCγ phosphorylates the ubiquitin tags on BMAL1, hence promoting cleavage and removal of these degradation signals from BMAL1(Zhang *et al.* 2012).

The role of BMAL1 in ubiquitination

BMAL1 (together with CLOCK) was found to associate with DDB1-CUL4, an important ubiquitin ligase in the DNA damage repair pathway. This association helps the monoubiquitination of histone H2B around E-box motifs near the *Per1* and *2* genes. This histone monoubiquitylation seems to be essential to the subsequent stable binding of the PER component of the PER/CRY complex, which results in the inhibition of CLOCK/BMAL1, and thus, completes the circadian cycle. This seems to be an additional safety layer to the circadian cycle's negative arm. Knocking down *Ddb1* or *Cul4* shortened circadian period. It was then proposed that other ubiquitinated histones might also be involved in similar processes of recognition and regulation of the other clock genes (Tamayo *et al.* 2015). This is an example of the non-degradative properties of ubiquitination within the clock machinery.

REV-ERBα

Ubiquitination

Like most of the other clock proteins, REV-ERBα is also ubiquitinated by an F-box protein, FBXW7, which selectively ubiquitinates and hence tags REV-ERBα for degradation (Zhao *et al.* 2016). Ubiquitination by FBXW7 requires prior phosphorylation of REV-ERBα by Cyclin dependent kinase 1 (CDK1). Cells with an siRNA based *Fbxw7* knockdown showed a reduction in the amplitude of circadian rhythms of a bioluminescent reporter. This is expected, since REV-ERBα would constitutively repress BMAL1 expression in the absence of degradation. Further, global deficits in sugar metabolism were observed, even in liver-specific *Fbxw7* KO mice (Zhao *et al.* 2016). *Fbxw7* expression is, interestingly, circadian, since it is controlled by D-binding protein (DBP), a rhythmically expressed transcription factor. SUMOylation seems to be a prerequisite for FBXW7-mediated ubiquitination of REV-ERBα (Pariollaud *et al.* 2018). CDK1 phosphorylation allows SUMO2 addition to REV-ERBα, which then allows for ubiquitination by FBXW7. In the lungs, this SUMOylation event seems to be activated by inflammatory signals. Thus, in the lungs, FBXW7-mediated ubiquitination of REV-ERBα seems to be primarily a response to inflammation (Pariollaud *et al.* 2018).

Arf-bp1 and PAM (Myc-bp2), a HECT E3 ligase and a RING-domain E3 ligase, respectively, act jointly to ubiquitinate and hence degrade REV-ERBα (Yin *et al.* 2010). Silencing their genes in HFR cells resulted in an upregulation in REV-ERBα levels and hence a downregulation of BMAL1, which resulted in a shorter period, as expected. It is thought that one of these ligases acts as a scaffold, while the other performs the catalytic activity, although the specific roles of the two have not yet been elucidated (Yin *et al.* 2010).

Seven in absentia 2 (SIAH2) is a RING type E3 ubiquitin ligase which was found to regulate REV-ERBα was via a screen aiming to isolate E3 ligases acting on this clock protein (DeBruyne *et al.* 2015). A FLAG-tagged version of this clock protein was expressed in AD293 cells, along with various ubiquitin ligases, protein synthesis was blocked, and the cells screened for a reduction of the FLAG signal. SIAH2 downregulated REV-ERBα, and its knockdown resulted in an increase in stability of REV-ERBα. This points towards a role for Siah2 as an E3 ligase that helps catalyze the degradation of REV-ERBα. However, SIAH2 is not itself the primary E3 ligase for this clock protein, as evidenced by the continued, efficient, if slow, degradation of REV-ERBα in the absence of SIAH2. Further, real-time bioluminescence recordings of *Bmal1*-luciferase cells showed a decreased amplitude and increased period upon knockdown of SIAH2 (DeBruyne *et al.* 2015). This increased period in the knockout of a ligase specific to REV-ERBα contrasts with the shortened period observed in cells knocked down for ARF-BP1 and PAM.

In addition to its role in CRY ubiquitination described earlier, FBXL3 contributes to the maintenance of circadian rhythms via a second mechanism, where it modulates the levels of REV-ERBα (Shi *et al.* 2013). Knocking out *Rev-erbα* rescues the long-period phenotype of *Fbxl3* KO mice, independently of the effect of the latter KO on CRYs and E box-mediated gene expression. In *Fbxl3* KOs, REV-ERBα levels remained constitutively high. Further, HDAC3, a histone deacetylase recruited by REV-ERBα to RREs (the DNA elements bound by REV-ERBα) to suppress transcription, showed a more sustained binding to chromatin in these KOs. Using binding and co-IP assays, it was shown that REV-ERBα recruits FBXL3 to the RREs and FBXL3 then inhibits transcriptional repression by ubiquitinating REV-ERBα and hence destabilizing the REV-ERBα:HDAC3 complex (Shi *et al.* 2013).

A diversity of roles for ubiquitination in circadian clocks

As can be seen from our overview of the literature, ubiquitination and deubiquitination serve a number of key roles in circadian clocks of different phyla. Although the factors involved are different, a few general principles can be observed.

Stability and period determination

As expected, E3 ligases and DUBs control the half-life of clock proteins and this helps in precisely setting the period of the circadian clock by modulating the stability of clock proteins. Based on when it takes place in the life cycle of a protein, modulating its half-life can have different effects. Pro-degradative ubiquitination signals in the build-up phase of the protein can prevent it from accumulating or peaking too fast. For example, FBXL21 ubiquitinates CRY proteins in the cytosol in the daytime and prevents them from peaking too early in the cycle (Hirano *et al.* 2013; Yoo *et al.* 2013).

On the other hand, in the phase out of the protein rhythms, any modification that destabilizes a protein will accelerate the circadian clock. FWD-1 in *Neurospora* (He *et al.* 2003) and FBXL3 in mammals (Busino *et al.* 2007) are good examples of E3 ligases promoting degradation of the proteins in the negative arm of the clock, hence allowing the restart of a new cycle of the circadian clock. This being said, these two phases of the clock protein regulation sometimes overlap in their mechanisms to regulate the speed of the clock: a fitting example of this is the crosstalk between FBXL21 and FBXL3, described above, where FBXL21 (which is also present in the nucleus) slows down the action of FBXL3 on CRY proteins (Yoo *et al.* 2013). Overall these effects on clock protein stability are key in regulating the free-running period of the clock.

Subcellular localization

While modulation of degradation pathways is the best-known function of ubiquitination, another role of ubiquitination is to regulate the intracellular localization of proteins. The clock is no exception to this, and ubiquitin tags can, in some cases, modulate subcellular localizations of clock proteins, as a way to modulate interactions within the clock machinery. One example relates to the TIM nuclear localization in *Drosophila*: CRY binds to and causes TIM to translocate into the nucleus, in response to light (Dissel *et al.* 2004), but the presence of E3 ligase JET prevents this translocation and rather targets TIM for degradation (Koh *et al.* 2006; Van Gelder 2006).

USP2, a mammalian DUB, also exerts a similar function. In *Usp2* KO fibroblasts, subcellular localization of PER1 was shown to be impaired (Yang *et al.* 2014). Moreover, the acrophase of the USP2 protein corresponds to that of the PER1 protein. This makes it possible that USP2 regulates nuclear localization of PER1, at a time of day when this clock protein generally regulates the activity of the CLOCK/BMAL1 complex, in conjunction with the CRYs.

Integration of input pathways

The processes of ubiquitination and deubiquitination can also mediate the connection between input pathways and the clock machinery. The most obvious example of this is the mediation of light input pathways. In many organisms, cryptochromes are light sensors and pass on this photic information to the clock. For example, in plants, COP1 is an E3 ligase which is activated by light input via CRYs (Yang *et al.* 2001). This causes ubiquitination (and degradation) of GI, which promotes transcription of CCA1 and LHY (Yu *et al.* 2008). Hence, light input in the daytime leads to the suppression of morning phased genes. Similarly, light is perceived by *Drosophila* CRY, leading to JET-mediated TIM ubiquitination and degradation, and hence, clock resetting (Koh *et*

al. 2006). In mammals, mice KO for the DUB USP2 showed alterations of the response of the clock to light stimulation (Yang *et al.* 2012).

The integration of input pathways by clock-based ubiquitination and deubiquitination also extends beyond photic inputs. An example is again provided by USP2: CRY1 protein is stabilized and upregulated in fibroblasts upon serum stimulation, due to its deubiquitination by USP2 (Tong *et al.* 2012). A similar USP2-dependent stabilization of CRY1 occurs in response to the cytokine TNFα, which could underlie some of the effects of inflammation on the molecular clock (Tong *et al.* 2012; Cermakian *et al.* 2014).

Activity of clock proteins

Another aspect of clock function that is modulated by ubiquitination is the activity of clock proteins. This is seen, for example, in the modulation of the transcriptional activity of the CLOCK/BMAL1 complex, independently of the regulation of their stability and abundance. Many PTMs regulate BMAL1 activity, including ubiquitination and SUMOylation. It is interesting to note that the ubiquitination of BMAL1, which closely follows its SUMOylation, occurs around the late subjective day and early subjective night, a time of the circadian cycle when CLOCK/BMAL1 activity is maximal (Lee *et al.* 2008). This might be related to the need for SUMOylation of BMAL1 for the recruitment of co-activator CBP by CLOCK/BMAL1 (Lee *et al.* 2015).

Regulation of clock output

Besides the timekeeping mechanisms and the input pathways, the other key component of any circadian clock consists in the output pathways, whereby the clock regulates cellular functions in

a rhythmic fashion. This is another aspect where ubiquitination and deubiquitination can be involved. Indeed, these processes, if they display circadian rhythmicity, can convey such rhythmicity to various cellular proteins.

This happens for substrates of USP2, which was discussed above as a regulator of input pathways to the clock but has roles to play in output pathways as well. Its abundance is rhythmic in all tissues studied so far, which suggest that it could rhythmically deubiquitinate multiple substrates across the body (Zhang *et al.* 2014; Yan *et al.* 2008). Examples of this process can be seen in the liver and in the small intestine. In the small intestine, USP2 regulates the expression of the membrane scaffolding protein NHERF4 and imparts a circadian rhythm on the abundance of this protein (Pouly *et al.* 2016). The authors suggested that this could lead to a rhythmic regulation of membrane permeability to calcium or of endocytosis. In the liver, USP2 was shown to regulate the daily glucose homeostasis (Molusky *et al.* 2012). This was proposed to happen through a regulation, by USP2, of the activity of the transcription factor C/EBPα, itself regulating HSD1, an enzyme involved in glucocorticoid signaling.

In other phyla, the involvement of ubiquitin-modifying enzymes in the circadian control of physiological processes includes, as described in previous sections, the action of USP12/13 on the timing of flowering in plants (Cui *et al.* 2013; Park *et al.* 2019; Lee *et al.* 2019). In *Neurospora*, the clock-controlled transcription factor CSP1 regulates the evening expression of various genes involved in particular in metabolism (Sancar *et al.* 2011; Sancar *et al.* 2015).

Looking past ubiquitination and deubiquitination

Interestingly, recent papers have shown a certain degree of redundancy to the process of clock protein degradation itself. Larrondo and colleagues showed, in *Neurospora*, that in the absence of

FWD1, the cognate E3 ligase of FRQ, the clock was still able to maintain its normal periodicity and amplitude (Larrondo *et al.* 2015). They showed, using mutants where they could modulate phosphorylation patterns without affecting protein stability, that the circadian cycle was modulated only by the availability of active, non-phosphorylated proteins. Upon phosphorylation, even in the absence of degradation, the clock machinery functioned normally, as if blind to the existence of the phosphorylated proteins (Larrondo *et al.* 2015; Kramer 2015). This could suggest that FWD1-mediated degradation of FRQ is dispensable for core clock function. Yet, evidence of altered conidiation cycles in FWD1-disrupted cells, reported above, points towards the importance of degradative signals in regulating circadian rhythms of clock output pathways, at the very least (He *et al.* 2003).

Either way, these data suggest that mechanisms beyond ubiquitination can be relevant for the fate of clock proteins and for the functioning of the clock feedback loops. This is not restricted to the *Neurospora* system and was observed in mammals as well. Ode and colleagues generated CRY mutants with point mutations of various phosphorylation sites on the protein. While in some of these mutants, the period lengthened, without altering the stability of the protein itself, in others the cells turned arrhythmic while the protein stability remained almost the same (Ode *et al.* 2017). This is consistent with a prior report where constant CRY1 and CRY2 in fibroblasts did not result in a loss of circadian rhythms, showing that CRY turnover and the cycling of these proteins is not the cornerstone of circadian-clock function in mammalian fibroblasts (Fan *et al.* 2007). Together, these results suggest that phosphorylation, which often precedes ubiquitination, might be able to independently control the clock. While it is undeniable that mutants of ubiquitin ligases and DUBs have shown altered circadian rhythms even though their precursor kinases were unaltered, it might be possible that some of the phosphorylated protein does not get ubiquitinated or degraded even

when the protein levels seem to be reducing during a circadian cycle. The protein might just be phosphorylated, sequestered and reused by the clock machinery at the phase when that protein is required yet again, in the circadian cycle.

Further credence is lent to this theory by the concept of Intrinsically Disordered Proteins (IDPs). These IDPs, which are multifunctional proteins, exist in a conformation which is not fully folded. Moreover, they can switch from one ensemble of conformations to another. This can be triggered by a PTM (e.g. phosphorylation), but such a switch can happen even without PTMs (Hurley *et al.* 2013). CRY proteins in mammals (Partch *et al.* 2005) and FRQ in *Neurospora* (Hurley *et al.* 2013) were shown to be IDPs. FRQ was shown to be degraded quicker when not bound to FRH (Cheng *et al.* 2005; Hurley *et al.* 2013). Such a regulation was proposed to be FWD-1 independent or to be "degraded by default", i.e. without the need for a chaperone or a ligase (Hurley *et al.* 2013). Therefore, IDP conformation switching and degradation by default appear to be additional potential ubiquitination-independent mechanisms of regulating the levels of clock proteins available to complete the clock cycle.

Finally, all of the above is not to undermine or play down the role of the processes of ubiquitination or deubiquitination in the clock. Rather, we use this opportunity to point out an additional layer of control to the clock machinery, which might be able to acutely shore up the circadian cycle, in the (temporary) absence of the ubiquitin-based degradation system. More research is required to understand this interplay between phosphorylation, IDPs and ubiquitin-based control of the clock mechanisms before actual conclusions about their relative importance. However, these concepts open up interesting and novel avenues, to look past the ubiquitin-proteasome system, about the regulation of the activity and life cycle of clock proteins.

The role of ubiquitination and deubiquitination in cancer

The circadian clock regulates virtually all physiological systems. Given the key roles of ubiquitination both in clock mechanisms themselves and in the cellular outputs from the clock, it is intuitive that ubiquitin-modifying enzymes active in the circadian system might mediate the links between circadian clocks and various disease conditions. Although this has not been explored extensively yet, examples include the possible involvement of the ubiquitin ligase UBE3A (which ubiquitinates BMAL1) in neurological disorders (Shi *et al.* 2015) and that of the DUB USP2 in metabolic pathways (Molusky *et al.* 2012). The area where links between ubiquitination in the clock and disease have been mostly drawn so far, though, is at the intersection of the cell cycle and cancer. Many studies have shown a definite link between the cell cycle and circadian rhythms (Borgs *et al.* 2009; Gaucher *et al.* 2018). This is also intuitively understandable, as the cell cycle is a strictly and precisely timed process in the lifetime of a cell. Hence, it comes as no surprise that cancers, which involve a dysregulation of the cell cycle, also involve the dysregulation of multiple processes implicated in the circadian clock pathway. Many of the ubiquitin-modifying enzymes mentioned above are also, hence, tumor suppressor genes and/or play a prominent role in regulating the cell cycle. Thus, studying the roles of the interacting partners of these ligases in a circadian context provides an opportunity to better understand the different pathways affected during cancer progression.

One example is FBXW7, an E3 ligase already mentioned above as an enzyme modifying REV-ERBα and possibly CRYs. It is also a tumor suppressor that regulates mammalian Target of Rapamycin (mTOR, a critical regulator of proliferation) expression. In tumors of the RenCa mouse renal carcinoma cell line, the expression of both FBXW7 and mTOR show a daily rhythm,

suggesting that ubiquitination of mTOR by this ligase confers it rhythmicity (in the liver, neither FBXW7 nor mTOR are rhythmic) (Okazaki *et al.* 2014).

While the study of FBXW7 led to finding therapeutic targets, sometimes the E3 ligases themselves are therapeutic targets. USP2, a circadian DUB described above as a regulator of CRY1, PER1 and BMAL1, was shown to deubiquitinate ErbB2, a gene associated with potentially fatal cases of breast cancer. By blocking degradation of ErbB2, USP2 promotes progression of the malignancy. This process seems to be accentuated due to the mis-regulation of USP2 rhythms, which lead to its constitutive activation throughout the day. Thus, targeting USP2 might represent a novel strategy to treat malignant breast cancer cells that express ErbB2 (Zhang *et al.* 2020).

In the same vein, anti-cancer drugs can also directly target ubiquitin tags in circadian pathways. The small molecule MLN4924 is a NEDD8-activating enzyme inhibitor, which has anti-tumoral properties. It is able to decrease ubiquitination of RORα, and thus, increase its stability (Zhang *et al.* 2016). Since RORα is an activator of *Bmal1* expression, the effect of MLN4924 on the expression of this gene was also tested: this drug increased *Bmal1* transcription and protein levels, an effect likely to be through RORα as the half-life of BMAL1 was unaffected. This induction of BMAL1 appears to be important for the anti-proliferative properties of MLN4924 (Zhang *et al.* 2016).

While on this topic, it is also interesting to remember that clock proteins themselves are involved in the degradation machinery of proteins involved in the cell cycle. For example, as described above, CRY2 is involved in regulating the ubiquitination of c-Myc and E2F, which controls cell proliferation (Huber *et al.* 2016; Chan *et al.* 2020). Also, PER2 regulates the degradation of p53, which is involved in cell apoptosis (Gotoh *et al.* 2014; Gotoh *et al.* 2015). Generally, the circadian clock has also been implicated in the control of various components of

the cell cycle (Masri and Sassone-Corsi 2018). Accordingly, it is unsurprising that defects in clock genes can affect cancer progression and circadian disruption is associated with an increased risk of cancer development: the reader is referred to other review articles for an in-depth discussion of the links between circadian rhythms, cell cycle and cancer (Soták *et al.* 2014; Masri and Sassone-Corsi 2018; Hernández-Rosas *et al.* 2020; Chan and Lamia 2020).

Finally, the administration of these anti-cancer drugs at various times of day might show contrasting levels of efficacy, based on the expression and presence of the target proteins. One example of this is the drug everolimus. Based on the rhythmic ubiquitination of mTOR by FBXW7, Okazaki and colleagues tested the effect of time of administration of everolimus, an mTOR-targeting drug. The treatment was found to be more effective and mouse survival improved upon treatment in the evening, as compared to treatment in the morning (with no change in the pharmacokinetics of the drug) (Okazaki *et al.* 2014). Another interesting example of chronotherapeutics is observed in the administration of cisplatin, a commonly used anti-cancer drug which works by damaging DNA in cancerous cells (Dasari and Tchounwou 2014). Cisplatin efficacy is limited by the availability of DNA repair mechanisms, which prevent cytotoxicity of healthy cells due to the damaging effects of the drug. XPA is a key component of the human excision repair complex, which can repair DNA damage resulting from cisplatin administration. Studies have shown that DNA repair rates are significantly different at different times of day (Kang and Sancar 2009; Kang *et al.* 2009). *XPA* expression has found to be strongly circadian and mediated by CRY1 mediated transcriptional activation, in some organs such as the liver (Kang *et al.* 2010). Similar to the other proteins having circadian expression discussed in this review, blocking HERC3, the cognate HECT domain containing E3 ligase of XPA1 increased the period

of XPA oscillations. Thus, timing of drug administration has become an important consideration when administering drugs such as cisplatin (Kang *et al.* 2010; Dakup *et al.* 2018).

In conclusion, we see that cancer therapeutics have been used to target downstream effectors of the ubiquitination/deubiquitination pathways, the pathways themselves and in some cases, they have been used to mimic this pathway. We also see how these degradative processes help us in increasing the efficacy of chronotherapeutic strategies against cancer. This informs us of the central role played by ubiquitination and deubiquitination in modulating cancer pathways, which makes them prime targets to combat this fatal disease.

Conclusion

In this review, we have provided an overview of clock mechanisms in eukaryotic models and the roles played by PTMs, particularly, ubiquitination and deubiquitination, in controlling the timing of these clocks. We see that these events, while mainly related to the control of the timing of degradation of the clock genes, are also able to control rhythms by other mechanisms such as assisting in binding to their target sites and histone ubiquitination, facilitating transcription of these proteins. Thus, we posit that the roles of ubiquitination and deubiquitination in the circadian system still require extensive study, especially in non-mammalian contexts. Even in mammals, indirect and non-degradative roles of these proteins still remain poorly understood. While recent research has shown that clock proteins themselves might have a role to play in the ubiquitination of other proteins, the specific mechanisms and contexts of these functions need further study. We have also seen how the importance of chronotherapeutics is beginning to become apparent. More work is required to untangle the roles of the different circadian players in the cancer progression pathways and to understand how this can be used in the context of combating the disease.

In conclusion, the diversity of roles played by ubiquitination-controlling proteins is best captured by the famous quote from William Shakespeare's play, *As you like it*, "All the world's a stage/ And all the men and women merely players: They have their exits and their entrances/ And one man in his time plays many parts". While we have seen the some of the parts played by these proteins and how they sequentially interact with the clock and each other, we have yet to see and understand all the roles these proteins might be playing in the circadian system.

Thus, while much work has been done, towards understanding the roles of ubiquitination and deubiquitination in the circadian clock, a lot of work remains to be done, before we can have a comprehensive idea of how all these proteins function, in conjunction to modulate the circadian system.

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The authors have no competing or financial interests to declare.

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Figure Legends

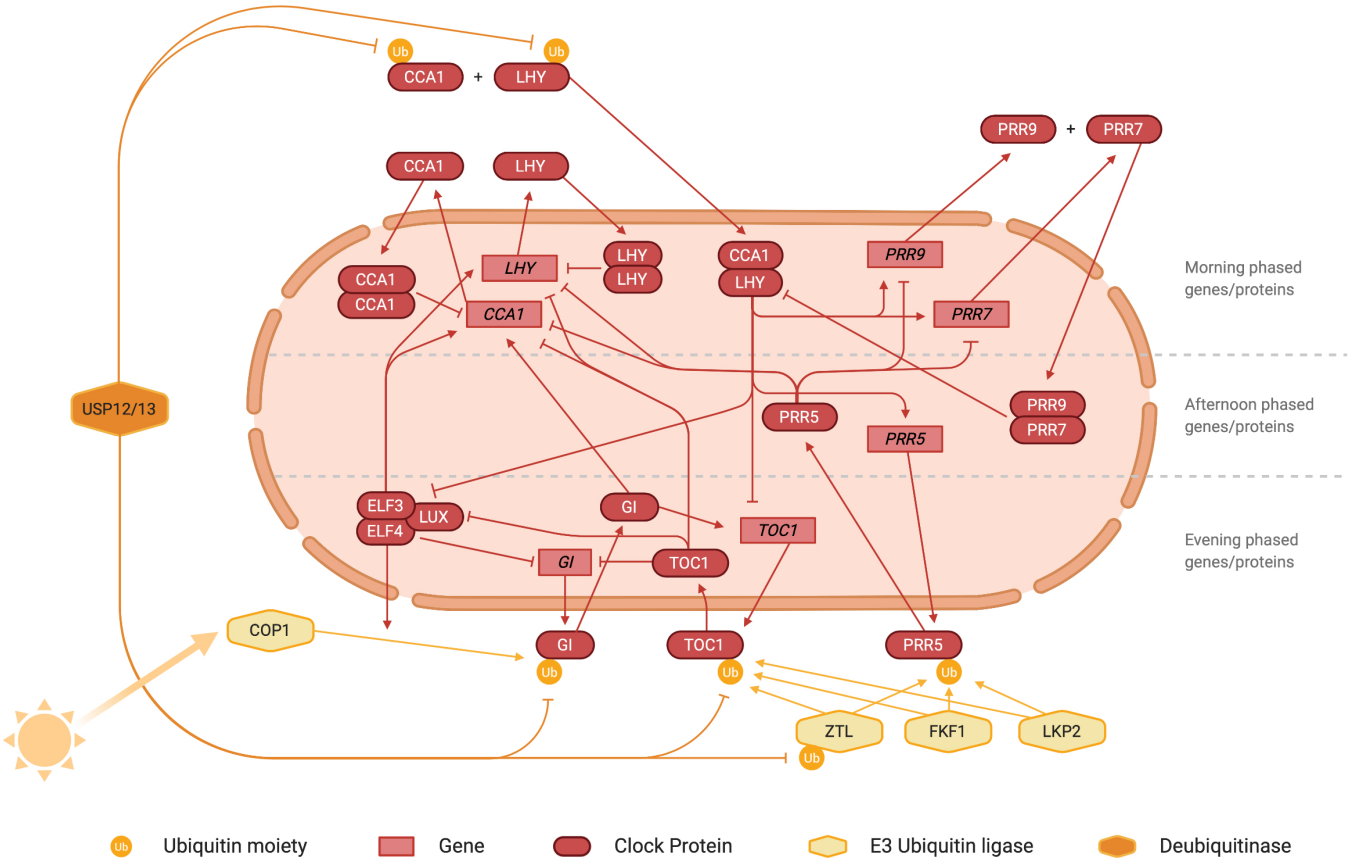
Figure 1. Schematic representation of the main components of the plant circadian clock, along with the E3 ubiquitin ligases and deubiquitinases known to regulate them. The central clock components CCA1 and LHY begin the clock cycle by activating the transcription of negative regulators, *PRR7* and *9*. The subsequent action of PRR proteins at various points through the day, negatively regulates the transcription of *CCA1* and *LHY*. A series of ubiquitination and deubiquitination events on the PRRs, in the evening/ early night regulate the precise timing of CCA1 and LHY accumulation again, hence restarting the circadian cycle.

Figure 2. Schematic representation of the main components of the *Neurospora* circadian clock, along with the E3 ubiquitin ligase known to regulate them. WC1 and WC2 associate to form the WCC and stimulate transcription of *frq* early in the subjective day. The FRQ protein homodimerizes and complexes with the helicase FRH, to inhibit the activity of the WCC. FWD1-mediated ubiquitination of FRQ at the end of this cycle halts the repression of the WCC, hence restarting the circadian cycle.

Figure 3. Schematic representation of the main components of the *Drosophila* circadian clock, along with the E3 ubiquitin ligases and deubiquitinases known to regulate them. CLK and CYC associate to stimulate transcription of *per* and *tim*. PER and TIM proteins then associate to repress the activity of the CLK/CYC complex. VRI and PDPε are proteins that constitute a secondary loop in this clock. They are activated by the CLK/CYC complex and they respectively repress and

activate *clk* expression. Competing ubiquitinating and deubiquitinating events on PER, TIM and CLK precisely regulate the timing of this clock.

Figure 4. Schematic representation of the main components of the mammalian circadian clock, along with the E3 ubiquitin ligases and deubiquitinases known to regulate them. CLOCK and BMAL1 associate to promote the transcription of *Cry* and *Per* genes. CRY and PER proteins then associate to repress the activity of the CLOCK/BMAL1 complex. The CLOCK/BMAL1 complex also transcribes *Rev-erb and Ror* genes. The REV-ERB and ROR proteins repress and activate the transcription of the *Bmal1* gene, respectively. Ubiquitination of various clock components affect their stability, nuclear localization and/or activity, hence regulating the timing of the circadian clock.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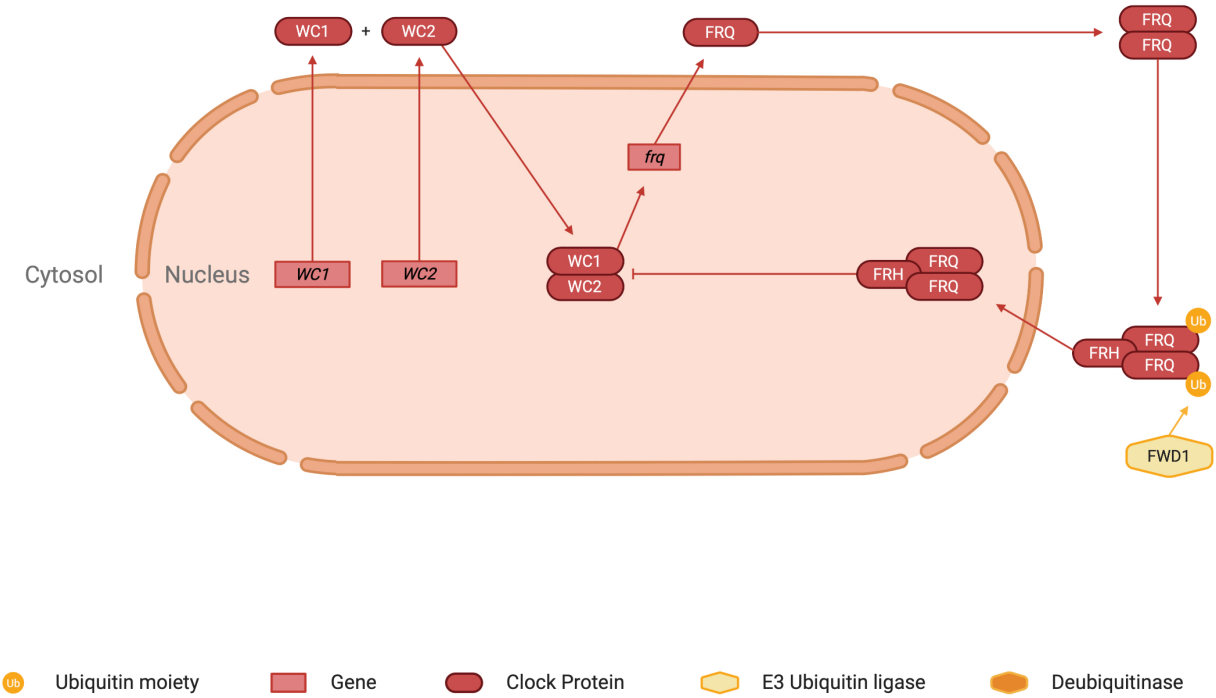
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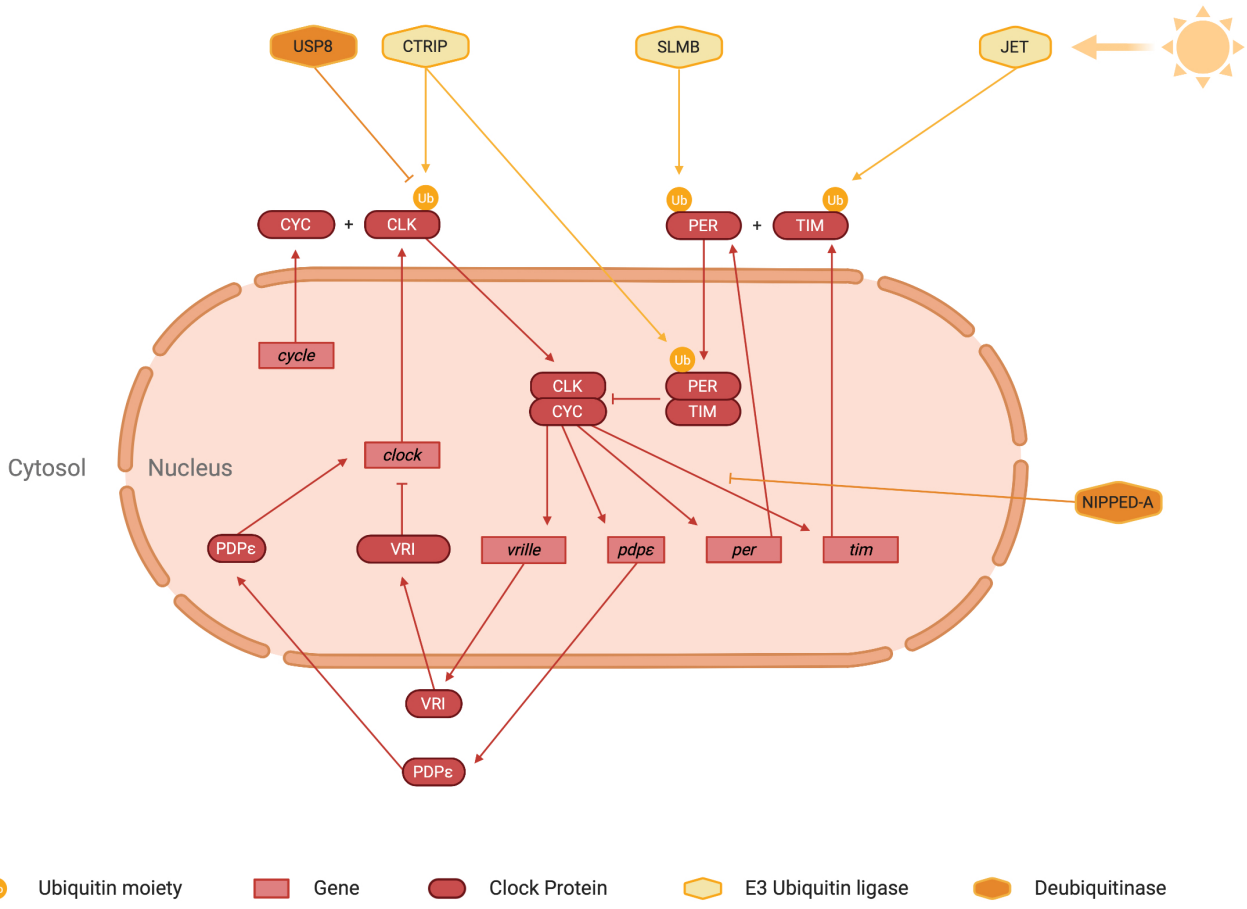
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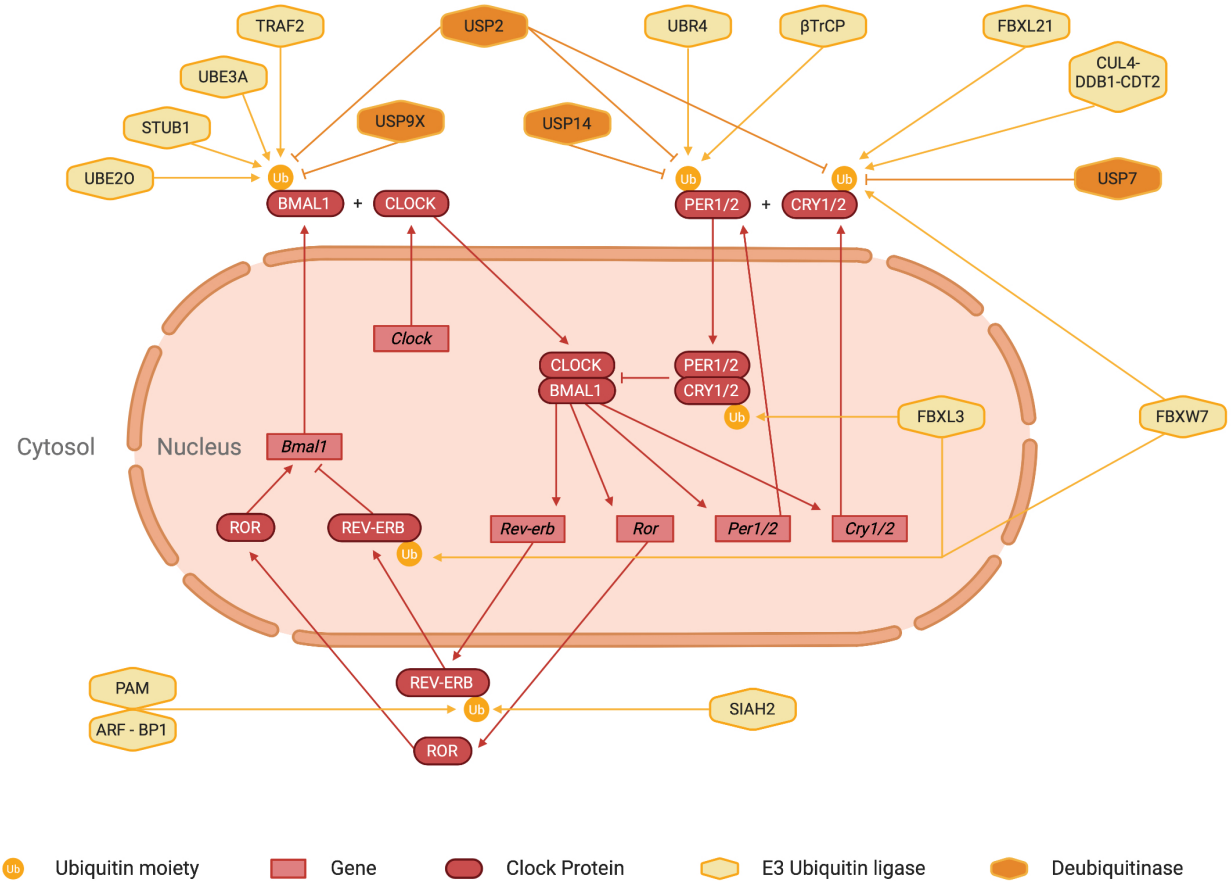
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