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DEUBIQUITINASES: KEY REGULATORS OF THE CIRCADIAN CLOCK

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ABSTRACT

All living organisms experience daily environmental cycles and have consequently evolved to synchronize and adapt to this changing environment. Biological processes such as hormonal secretion, body temperature and sleep follow daily cycles called circadian rhythms that are driven by a molecular clock running in most cells and tissues of the body. This clock is composed of transcriptional-translational negative feedback loops involving clock genes and proteins. This molecular mechanism functions with a period of ~24 hours, and it promotes daily rhythms in the expression of numerous genes. For this robust mechanism to function, the abundance and activity of clock proteins need to be tightly regulated. One of the mechanisms by which this can be achieved is ubiquitination. Indeed, many ubiquitin ligases can tag core clock proteins in order to target them for proteasomal degradation. However, deubiquitinases can reverse this process by removing or modifying these ubiquitin signals and are thus important enzymes in clock protein homeostasis and regulation. Recent studies on the mammalian and *Drosophila* clock mechanisms have identified a number of deubiquitinases able to stabilize core clock proteins, change their cellular localization or even regulate their activity. In this review, we aim to discuss the fundamental roles of ubiquitination and deubiquitination in the circadian clock by presenting all deubiquitinases found to be involved in circadian rhythms in the aim to give a global view of recent advances in this emerging field.

INTRODUCTION

Circadian rhythms are daily physiological changes that allow living organisms to anticipate and synchronize with daily environmental variations such as the light-dark cycle (1). These rhythms can be observed in a variety of processes, ranging from hormonal secretion to sleep and body temperature (2). In fact, circadian rhythms are both entrainable and endogenous, meaning that they are regulated by external cues such as light and food intake, but also by an autonomous oscillator within the organism (2). The neuronal master clock, located in the brain in the suprachiasmatic nucleus (SCN), can integrate light inputs to transmit precise timing information to other clocks throughout the body (3).

At the tissue and cellular level, circadian rhythms can be observed in the ~24-hour rhythmic expression of thousands of genes. These molecular oscillators are driven by a clock mechanism running in almost every cell of the body (**Figure 1**) (4). This clock is composed of clock genes and proteins interacting in transcriptional-translational negative feedback loops (2). In the positive arm of the cycle, the transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK) (or its paralog Neuronal PAS Domain Protein 2 (NPAS2)) and Brain and Muscle ARNT-Like 1 (BMAL1) heterodimerize and activate the transcription of many genes including the clock genes *Cryptochrome* (*Cry1/2*) and *Period* (*Per1/2/3*) (5, 6). Once translated, the CRY and PER proteins form complexes and translocate into the nucleus where they inhibit their own transcription through the repression of CLOCK/BMAL1 activity, which constitutes the negative arm of the feedback loop (3). CLOCK/BMAL1 also activate genes encoding the orphan nuclear receptors REV-ERBα/β and RORα/β/γ, which in turn repress and activate, respectively, the transcription of *Bmal1*, thereby forming additional regulatory loops to provide further robustness to the clock (7-9).

The timely degradation of clock proteins is essential for the cycle to proceed at the right pace and to restart every day, and for the rhythmic expression of CLOCK/BMAL1-regulated transcripts.

Indeed, it is now known that most clock proteins are modified at a post-translational level, which regulates their stability, cellular localization and activity (5, 10, 11). Post-translational modifications (PTMs) of clock proteins such as phosphorylation, acetylation, poly ADP-ribosylation, O-GlcNAcylation, SUMOylation and ubiquitination have been extensively studied, and their proper balance is critical to maintain protein homeostasis in the clock (10-12). It is therefore essential to not only identify enzymes catalysing these reactions, but also the ones that oppose and balance them.

The counteracting processes of deubiquitination and ubiquitination center around a small 8.5 kDa protein called ubiquitin. The covalent attachment of a single ubiquitin or a ubiquitin chain to a protein can determine its activity level or localization of the protein but generally, it targets the protein for degradation by the proteasome (13). Deubiquitinating enzymes (or deubiquitinases) can remove and modify these ubiquitin signals to reverse this process and promote protein stability (or other aspects of the protein life cycle) (5, 14). Thus, deubiquitinases might be important regulators of the clock given that they can maintain precise levels of clock proteins and prevent their premature degradation. This review aims to summarize the current knowledge on the deubiquitinases found to have a regulatory role in the clock.

THE IMPORTANCE OF UBIQUITINATION IN THE MAMMALIAN CLOCK

The process of ubiquitination can precisely determine the half-life, localization and activity of various clock proteins to accurately maintain the periodicity of the circadian feedback loop (5, 15). In short, the process of ubiquitination is generally mediated by the cohesive action of three different groups of proteins: The E1 ubiquitin-activating enzyme activates ubiquitin for conjugation using ATP. The E2 enzyme then thiolates the activated ubiquitin and transfers it to the E3 ubiquitin-conjugating enzyme, which binds to the target protein and ubiquitinates it (16).

The enzymes ubiquitinating some of the clock proteins have been identified and are briefly described in this section. For more details on the role of ubiquitination in the clock, please refer to recent reviews on this topic (5, 15).

The first known ubiquitinating enzymes of the CRY proteins were the F-Box and Leucine-Rich Repeat Protein 3 and 21 (FBXL3 and FBXL21). During the daytime, FBXL21 stabilizes CRY proteins in the cytoplasm and regulates their accumulation (17). Later in the cycle, FBXL21 and FBXL3 can both act on CRYs in the nucleus; however, FBXL21 has a high affinity for the CRYs, but low activity, while FBXL3 has lower affinity, but tags them for degradation much faster (18). Hence, the spatiotemporal distribution of these ubiquitin ligases is central for their antagonistic regulation of the accumulation, degradation and thus, repressive role of CRY proteins within the clock feedback loop (17-21). Further, roles for F-box and WD repeat domain-containing 7 (FBXW7) and Cullin 4-Damaged DNA-Binding Protein 1 (CUL4-DDB1) in ubiquitinating CRY proteins have also been proposed to act in the mammalian clock context (22, 23).

The PERIOD proteins have the shortest half-life among clock proteins (24) and hence, are heavily regulated by PTMs. Beta-Transducin Repeat-Containing Protein (β-TrCP1 or FBXW1 and β-TrCP2 or FBXW11) are the primary ubiquitin ligases of the PER proteins (24-28). β -TrCP1 and β-TrCP2 were shown to be essential to maintain the amplitude and period of circadian oscillations in mouse fibroblasts (24, 26, 29). Furthermore, mice heterozygous for both *β-Trcp1* and *β-Trcp2* show a lengthened period of locomotor activity rhythms (24). Ubiquitin protein ligase E3 component N-recognin 4 (UBR4) was identified as an additional ubiquitinating protein associated with PER2 (30). Recently, UBR4 was also shown to have a role of modulating neuropeptide release in SCN neurons, hence affecting network synchrony (31).

Ubiquitin Protein Ligase E3A (UBE3A), Ubiquitin-Conjugating Enzyme E2 O (UBE2O), TNF Receptor-Associated Factor 2 (TRAF2) and STIP1 homology and U-box-containing protein 1 (STUB1) are all ubiquitin ligases found to ubiquitinate BMAL1 and to promote its degradation in cell-based assays (32-35). Knocking out UBE3A or TRAF2 in cells increased the amplitude and period of cellular rhythms (32, 34). UBE3A is expressed in neurons of the SCN (36, 37), and it interacts with BMAL1 and potentially destabilize it *in vivo* (37). Moreover, mice heterozygous for a null mutation of the *Ube3a* gene display altered circadian behavior (37). However, there was remaining UBE3A expression in the SCN of these mice, possibly due to incomplete imprinting of the paternal *Ube3a* allele within this brain structure (36, 38). Hence, more studies are needed to determine the exact role of UBE3A in the circadian system. As for TRAF2, UBE2O and STUB1, they are yet to be studied in the context of the mammalian circadian clock *in vivo*.

FBXW7, in addition to its possible action on CRYs, can affect REV-ERBα levels. While whole body KOs were embryonic lethal, hepatic KO showed altered clock gene expression in the liver (39). SIAH2 was another protein found to increase degradation of REV-ERBα, and *Siah2* knockdown cells show an increased period of circadian rhythms of a bioluminescent reporter (40). Sp1A/Ryanodine receptor domain and SOCS box-containing 1 and 4 (SPSB1 and SPSB4) also both decrease REV-ERBα stability and accordingly, their knockdowns lengthen the period of bioluminescence rhythms (41). Additionally, the E3 ubiquitin ligases ARF-binding protein 1 (AFR-BP1, also called HECT, UBA and WWE domain-containing protein 1 or HUWE1) and protein associated with Myc (PAM, also called Myc-binding protein 2 or MYCBP2) together promote the ubiquitination of REV-ERBα and lead to its rapid degradation (42).

With so many proteins determining the timing of degradation of circadian clock components, it becomes imperative to also understand the counteracting process of deubiquitination to completely understand the role of ubiquitin as a PTM in the circadian clock.

DEUBIQUITINASES AND THE CLOCK

The human genome encodes around a hundred deubiquitinases (14). Although they can be divided in seven families based on their structure, ubiquitin specific peptidases (USPs) make up nearly half of them with more than 50 members (14). Mainly studied in the context of cell cycle and cancer (43), deubiquitinases are now known to participate in other cellular functions including the circadian clock (5). The following sections will explore all deubiquitinases that have been identified so far as regulators of clock proteins.

USP2

USP2 has been the most studied deubiquitinase in the context of circadian clocks so far. It was first found to be encoded by one of the rare genes that are rhythmically expressed in most tissues of the body, including the SCN (4, 44-48). Deletion of *Usp2* gene in mice led to alterations in the expression profiles of clock genes in primary fibroblasts, the SCN and the liver (45, 49). Subsequent work provided further evidence that USP2 could regulate the clock via its action on clock proteins such as PER1, CRY1 and BMAL1 (45, 50, 51), although it was only shown to interact directly with PER1 (50).

USP2 has a few isoforms that may have differential roles in the clock. The expression of *Usp2b*, the shorter USP2 isoform, displays more robust rhythms than *Usp2a* in the SCN, the liver and the retina (45). Interestingly, USP2b was suggested to be in complex with BMAL1 and PER1

in the SCN and was shown to regulate BMAL1 stability through its deubiquitination (45, 52). USP2a was rather shown to have a similar effect on CRY1. Indeed, CRY1 ubiquitination and degradation was enhanced when *Usp2a* was knocked down both *in vitro* and in mouse livers (51). Accordingly, its overexpression had the opposite effect (51). It is however not clear whether these effects are strictly isoform-specific, as these studies focused exclusively on one isoform and did not show results for the other.

Although ubiquitin modifications are thought to mainly impact proteasome-dependent degradation, they can also modify the cellular localization of targeted proteins. For example, USP2-mediated PER1 deubiquitination was not found to alter its degradation rate, but rather its nuclear localization (49, 50). For instance, cellular overexpression of either USP2a or USP2b increased the nuclear retention of PER1, which then led to a greater repression on CLOCK/BMAL1 transcriptional activity (49). Consistently, the loss of USP2 in mouse primary fibroblasts and livers increased the amount of cytoplasmic PER1 and significantly dampened *Per2* and *Rev-erbα* rhythmic expression (49).

These multiple roles of USP2 in the regulation of circadian rhythms found both *in vitro* and *in vivo* suggested that the loss of USP2 would also impact circadian behaviors. Indeed, mice lacking USP2 had a slight increase in the free running period of locomotor activity rhythms measured in constant darkness, although only one study found the difference to be statistically significant (45, 50) . These KO mice were also consistently shown to have increased wheel running activity in a 12h:12h light-dark cycle, and further analysis revealed less activity fragmentation than wildtype (45, 50, 53). More interestingly, the clock of USP2 knockout mice showed a slower entrainment to phase advances of the light-dark cycle, but adapted more rapidly than the clock of wildtype mice to a phase delay. This suggested a key role for USP2 in the light response pathway (50). Consistent

with this, light pulses of various lengths at the beginning of the night led to greater delays of activity rhythms in USP2 knockout mice (45, 50). Although it is clear that USP2 regulates the clock, further investigation is needed to understand how it can modulate light response and to identify its possible roles in other clocks in the body.

Furthermore, USP2 might be involved in the clock outputs to generate rhythmicity in various cellular pathways. Its rhythmic expression in many tissues can lead to a rhythmic pattern of deubiquitination and thus rhythmic regulation of many targets. In the liver, USP2 was found to regulate glucose homeostasis in a circadian manner. It was shown to deubiquitinate and modulate the transcriptional activity of the CCAAT-enhancer-binding protein C/EBPα in hepatocytes, which in turn controls the expression of 11β-Hydroxysteroid dehydrogenases type 1 (HSD1), an important enzyme in glucose metabolism (54). USP2 also found rhythmically regulates calcium homeostasis in the small intestine. In fact, USP2 negatively regulates the levels of the PDZ-domain containing scaffolding protein NHERF4 involved in calcium absorption (55). Taken together, these findings confirm that USP2 can act as an output regulator to transmit timing information from the circadian clock to other physiological processes.

USP7

USP7, also called Herpes virus-associated ubiquitin-specific protease or HAUSP, is also thought to play a regulatory role in the clock. It can interact with both CRY1 and CRY2 (more strongly with the former) (56, 57). Similar to USP2, USP7 deubiquitinates and stabilizes CRY1 (56-58). However, the specific effects of USP7 on circadian rhythms is unclear. Bioluminescence rhythms following either the cellular knockdown or overexpression of *Usp7* resulted in inconsistent phenotypes. First, reducing USP7 activity both through RNA interference and

pharmaceutical inhibition lengthened the period of cellular rhythms in both immortalized mouse embryonic fibroblasts (MEFs) and U2OS cells, a human osteosarcoma cell line commonly used in the circadian field because of its robust rhythms (56). However, the opposite effect was found one year later in MEFs and NIH3T3 mouse fibroblasts (57), where its knockdown shortened the period of cellular rhythms while its overexpression had a lengthening effect (57). These later results are more consistent with previous studies in which the reduction in CRY1 levels decreased the period of both cellular rhythms and mice locomotor activity rhythms (59-61). However, the discrepancies between these studies might come from a differential interaction of USP7 with CRY1 and CRY2, as the knockdown of the latter is thought to lengthen the period of cellular rhythms (59, 61). USP7 might also act in a tissue-specific manner or might be redundant with other DUBs, all of which could impact the phenotypes obtained in diverse cell lines.

It is clear that USP7 plays a role in stabilizing the negative arm of the molecular clock but its regulation might also involve other modulators. USP7 activity can be negatively regulated by melanoma-associated antigen gene (MAGE) protein family member L2 (MAGEL2) (58), an E3 ubiquitin ligase regulator whose disruption is associated with sleep disorders in human and circadian rhythms disruptions in mice (62-64). Interestingly, MAGEL2 is highly expressed in the SCN (65) and was shown to interact with CLOCK/BMAL1 to repress their transcriptional activity (66). These findings suggest that USP7 might be modulating the clock as part of a larger complex, and it would be interesting to investigate how the balance of these regulators is achieved and how it can influence circadian rhythms.

Other deubiquitinases involved in the mammalian clock

The DUB USP9X appears to regulate the positive arm of the molecular clock through its interaction with BMAL1 (67). By deubiquitinating BMAL1, it increases its stability and levels (67). Accordingly, decreasing USP9X levels though RNA interference led to reduced expression of BMAL1 target genes *Per2* and *Cry1* in a mouse neuroblastoma cell line (67). However, a similar decrease in *Usp9x* expression in U2OS cells only led to a slight decrease in the amplitude of cellular rhythms and no change in period (67). It is unclear as to why *Usp9x* knockdown did not result in a more striking loss of cellular rhythms, since the knockdown of BMAL1 itself was shown to lead to either arrhythmicity or major reduction in amplitude (59, 68, 69). These discrepancies might arise from a tissue-specific interaction between USP9X and BMAL1. Alternatively, other redundant DUBs might be able to rescue the loss of USP9X in certain tissues and cell lines, but not in others. It is thus clear that future research on USP9X will be necessary to understand its role at a tissue level and how it can be interconnected with other clock regulators.

Although it is not yet known if USP14 interacts directly or indirectly with PERIOD proteins, it was suggested to reduce their polyubiquitination to prevent their downregulation (24). PER proteins have very short half-lives (70) and the tight regulation of their degradation is critical to maintain the 24-hours pace of the cellular clock. Indeed, the expression of a dominant negative form of USP14 in HEK293a cells led to a reduction in the abundance of both PER1 and PER2 (24). Similarly, this treatment in MEFs resulted in a reduced PER2 half-life and a dose-dependent decrease in the period of cellular rhythms (24).

UCHL1 is a member of the Ubiquitin C-terminal Hydrolase or UCH family of deubiquitinases, which are structurally and catalytically different from all deubiquitinases introduced so far (14). Indeed, UCHL1 was suggested to recycle ubiquitin and maintain cellular ubiquitin homeostasis (71). Furthermore, UCHL1 is one of the most abundant protein in the brain and is almost

exclusively expressed in neurons (72). Its transcript was also detected in high levels in the SCN (73). Interestingly, mice with a spontaneous mutation in the *Uchl1* gene, called gracile axonal dystrophy or gad mice, display disrupted circadian behavior both in a 12h:12h light-dark cycle and in constant darkness (74). These mice also have trouble adjusting their locomotor activity rhythms to a phase delay of the light-dark cycle and they seem more active during the daytime compared to wildtype mice (74). Of note, given that these mutant mice exhibit motor defects, it is not clear whether the phenotypes are strictly due to circadian disruption or sleep disturbances. Indeed, UCHL1 might be involved in a multitude of neuronal processes, given its high expression throughout the brain. Nevertheless, considering these findings, it would be interesting to investigate further the role of UCHL1 in the clock.

Deubiquitinases involved in the *Drosophila* clock

The basic features of the circadian clock are conserved between animal phyla. Indeed, the *Drosophila* clock mechanism is also based on negative transcriptional-translational feedback loops alike those in mammals. Genetic screens in fruit flies have been central to the discovery of core clock components and of the clock feedback mechanism (75). A CLOCK/CYCLE complex activate the transcription of clock genes such as *timeless (tim)* and *period (per).* Once translated, PER and TIM heterodimerize to inhibit CLOCK/CYCLE activity (76). Additional regulatory loops involve the transcription factors PAR domain protein 1ε (PDP1ε) and VRILLE (VRI), whose genes are also under the control of CLOCK/CYCLE: PDP1ε activates the transcription of *clock*, while VRI inhibits it (**Figure 2**) (77).

Many of *Drosophila* clock proteins are known to be ubiquitinated by various ubiquitin ligases. Notably, PER and TIM degradation in constant darkness, but not in a light-dark cycle, is modulated

by the F-box supernumerary limbs (SLIMB) ubiquitin ligase (78). Consequently, a mutation in the *Slimb* gene resulted in arrhythmicity when flies were put in constant darkness (78). TIM can also be ubiquitinated by the E3 ligase JETLAG, a reaction that is involved in the *Drosophila* light response pathway, as JETLAG mutation led to a reduction in light-induced phase shifts of activity rhythms (79).

The positive arm of the circadian clock can also be regulated by ubiquitination. The HECT domain family E3 ubiquitin ligase circadian TRIP (CTRIP) ubiquitinates and destabilizes CLOCK, and *ctrip* deficiency led to an increase in the expression of most clock genes (80). These findings provide evidence that ubiquitination can largely impact the *Drosophila* clock mechanism. However, little is still known about the role of deubiquitination in regulating clock proteins.

The first appearance of USP8 in the chronobiology field was in the early 2000s when its transcript was found to be rhythmically expressed in the *Drosophila* brain (named CG5798 at the time) and more specifically in clock neurons (81, 82). However, no further studies looked at its role in the clock until later on, when Luo et al. (83) knocked down *Usp8* in flies. They found an increased expression of CLOCK/CYCLE target genes, including *per* and *tim*. In fact, USP8 directly deubiquitinates CLOCK, which inhibits its transcriptional activity, thereby regulating the transcription of many clock-controlled genes (83). At the behavioral level, these flies were either arrhythmic or had a longer period of locomotor activity rhythms (83). Unfortunately, the embryonic lethality of *Usp8* knockout in mice (84) could make the study of mammalian USP8 less straightforward. A more targeted deletion of USP8 could be used to investigate its role in the mammalian clock.

The SAGA (Spt-Ada-Gcn5 acetyltransferase) co-activator is a large multi-module complex involved in chromatin modification and in transcriptional regulation (85). In *Drosophila*, its DUB

module includes the deubiquitinase NON-STOP (NOT) (85). Interestingly, knocking down *not* in clock cells led to a lengthening of the period of locomotor activity rhythms as well as a decrease in their robustness (86). Many *Drosophila* core clock genes (*tim*, *per*, *pdp1ε*, *vri, clock* and *cycle*) had a reduced expression in the head of these flies (87). Additionally, *not* deficiency led to a significant increase in the level of histone 2B ubiquitination at *tim* and *pdp1ε* loci (87).

Not activity could be mediated by NIPPED-A, an adaptor protein involved with the SAGA complex. In fact, *Nipped-a* knockdown specifically in clock neurons led to a lengthening of locomotor activity rhythms and the double knockdown of both *not* and *Nipped-a* amplified this phenotype (86, 87). Taken together, these findings suggest that the DUB module of the SAGA complex involving *not* can promote the CLOCK/CYCLE-mediated transcription through the deubiquitination of histone 2B at clock gene loci, and this process is thought to be facilitated by NIPPED-A. Regarding the mammalian clock, NOT homologue, USP22, have yet to be linked to clock regulation. However, histone 2B ubiquitination at mammalian clock gene loci was found to regulate clock gene transcription in mouse livers (88). These results support a broader role for deubiquitination in circadian rhythms regulation beyond their direct impact on clock proteins.

CONCLUSION

In this review, we discussed all studies published so far on clock-regulating deubiquitinases to provide a better understanding of the role of deubiquitination in circadian rhythms. Studies on USP2 have established its important role in the robustness, period determination and light response of mammalian circadian rhythms. As for USP7, *in vitro* work allowed validating its clock protein targets. However, the lack of consistency between phenotypes of different studies is confusing with regards to its precise effect on cellular rhythms. In the case of other deubiquitinases, more

research is needed to understand their level of implication in circadian rhythms. Additionally, more *in vivo* studies would be necessary to better define the role of deubiquitination in circadian rhythms. The discovery of clock mechanisms has largely benefitted from a synergy of work on different models, e.g. rodents and *Drosophila*. The same could be true for DUBs. When orthologs to mammalian DUBs exist, fruitfly studies could allow efficient genetic studies. *Drosophila* genetics could also lead to the discovery of new DUBs important for the clock. Conversely, the potential role of mammalian homologs of DUBs found to have a role in *Drosophila* could be investigated.

While the research summarized here has identified a few clock-regulating deubiquitinases, with more than a hundred deubiquitinating enzymes encoded in the human genome, there is probably more to identify. As more protein modifying enzymes are found to regulate clock function, it becomes imperative to understand their interplay. It is now known that not only the combinations of PTMs, but also the sequential modification of clock proteins, are essential to precisely regulate circadian rhythms. For example, PKCγ-mediated phosphorylation of BMAL1 was shown to decrease its ubiquitination and to lead to its stabilization (89). Additionally, SUMOylation of BMAL1 by SUMO2/3 leads to its ubiquitination and further proteasomal degradation, and this is associated with the time of highest transcriptional activity of the CLOCK/BMAL1 complex (52, 90). But the complexity of PTMs in the clock is also due to variations amongst each type of signal. For instance, proteins can either be mono- or polyubiquitinated, which is thought to have differential effects on the target protein, whether it is a change in its cellular localization, activity, stability or even changes in the recognition of ubiquitin-binding domains of other enzymes (91). It will therefore be necessary in future research

to not only identify key ubiquitin ligases and DUBs, but also the specific ubiquitin code that they leave on each of their clock target and the proteins that can interact with it.

Overall, although this field of study is still in its early stages, we believe that there is enough evidence to confirm deubiquitination as an important regulator of the circadian clock. Future research will identify more clock-regulating deubiquitinases and delineate their roles beyond cellular rhythms, for example in tissue-specific clocks or in circadian behavior.

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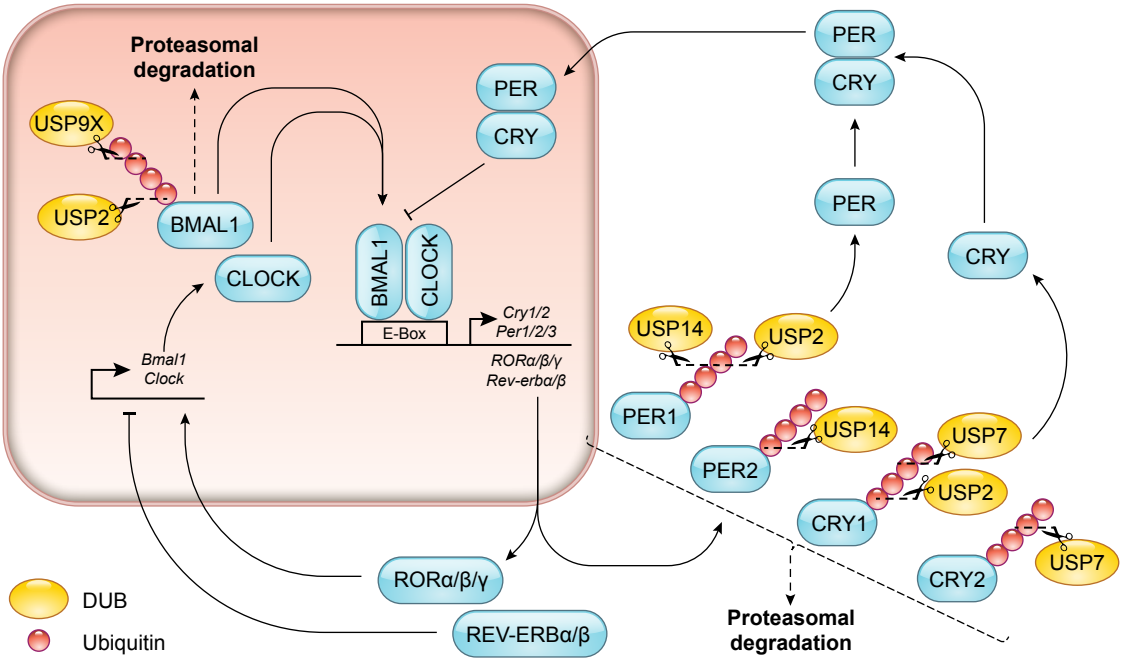
FIGURE LEGENDS

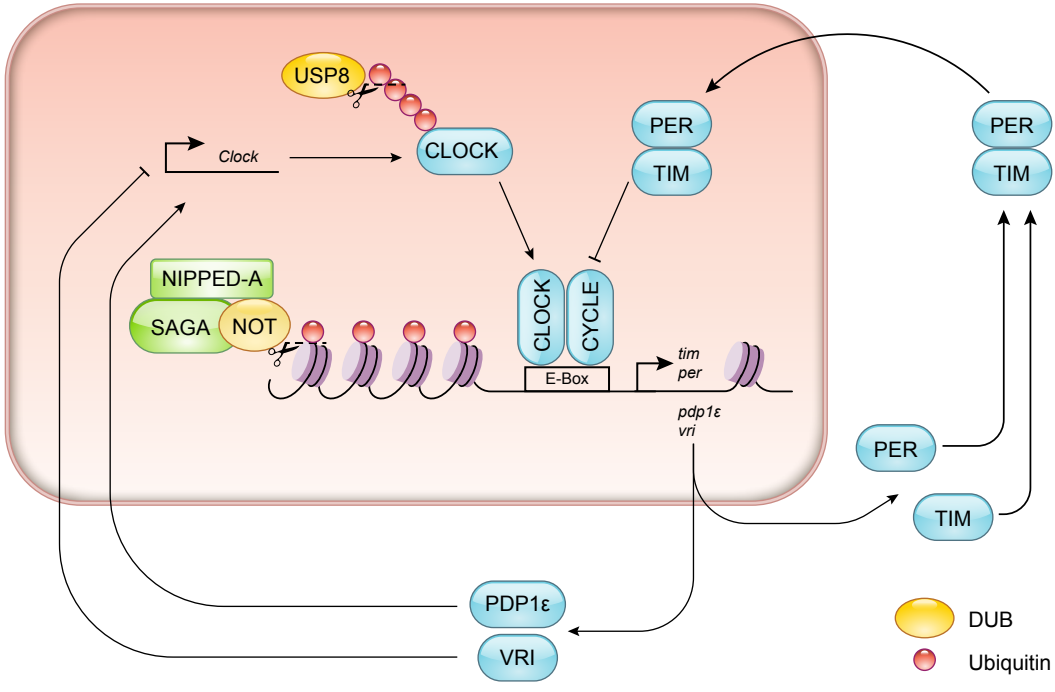
Figure 1. Deubiquitinases involved in the mammalian circadian clock.

The mammalian molecular clock is composed of daily negative transcriptional-translational feedback loops. By binding to E-box elements, CLOCK/BMAL1 activates the transcription of clock genes such as *Periods (Per1/2/3), Cryptochromes (Cry1/2) and* nuclear receptors *Rev-erbα/β* and *RORα/β/γ*. PER and CRY proteins then translocate into the nucleus to inhibit CLOCK/BMAL1 activity. Proteasomal degradation of PERs and CRYs relieves this repression to allow the cycle to restart. REV-ERBα/β and RORα/β/γ provide further robustness by regulating *Bmal1* and *Clock* expression. USP2 and USP9X deubiquitinate BMAL1 to increase its stability and promote clock gene expression. USP7 and USP2 deubiquitinate CRY proteins, which results in their increased stability. USP14 has a similar effect on PER proteins by decreasing their polyubiquitination. Finally, USP2 was shown to interact directly with PER1 and to enhance its nuclear translocation through its deubiquitination.

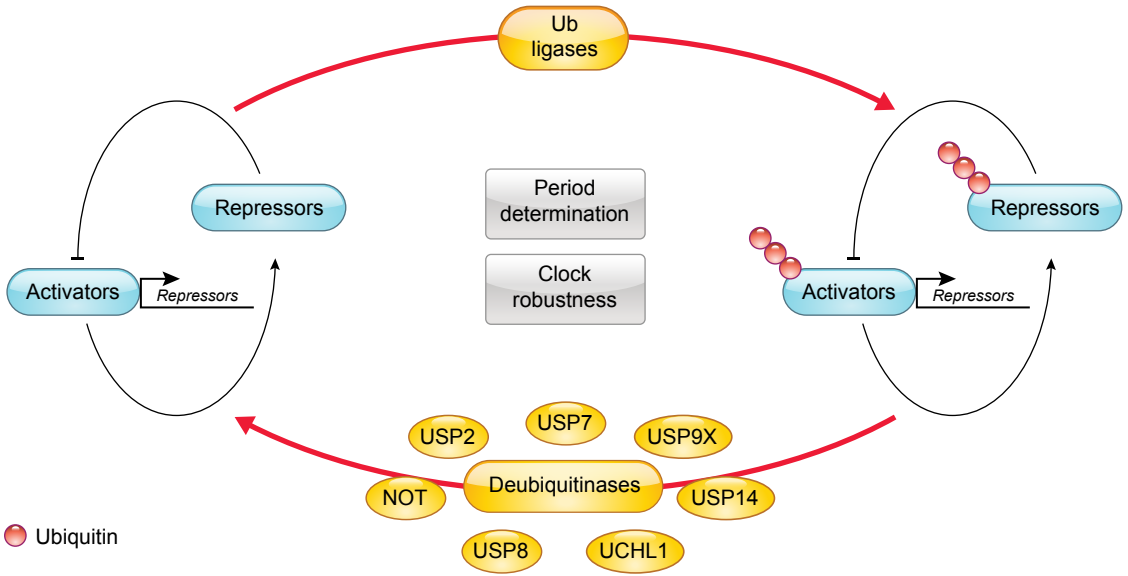
Figure 2. Deubiquitinases involved in the *Drosophila* circadian clock.

In the *Drosophila* molecular clock mechanism, CLOCK/CYCLE binds E-box elements in the promoter region of clock genes such as *tim, per, pdp1ε and vri* to activate their transcription. PER and TIM proteins then heterodimerize to translocate into the nucleus and repress CLOCK/CYCLE. Additional loops involve the transcription factors PDP1ε and VRI, which respectively activate and repress *Clock* expression. USP8 decrease the transcriptional activity of CLOCK through its deubiquitination. Furthermore, a deubiquitinase part of the SAGA DUB module, called NOT, deubiquitinates histone 2B at both *tim* and *pdp1ε* loci in order to promote their transcription. This process is also thought to be facilitated by the adaptor protein NIPPED-A.


Proteasomal degradation
USP9X
USP2
BMAL1
CLOCK
PER
CRY
BMAL1
CLOCK
E-Box
Cry1/2
Per1/2/3
RORα/β/γ
Rev-erbα/β
Bmal1
Clock
RORα/β/γ
REV-ERBα/β
PER
CRY
PER
CRY
USP14
USP2
PER1
PER2
USP14
USP7
USP2
CRY1
CRY2
USP7
Proteasomal degradation
DUB
Ubiquitin


USP8
CLOCK
Clock
PER
TIM
NIPPED-A
SAGA
NOT
CLOCK
CYCLE
E-Box
tim
per
pdp1ε
vri
PER
TIM
PER
TIM
PDP1ε
VRI
DUB
Ubiquitin

The balance between ubiquitination and deubiquitination in regulating the circadian clock



The circadian clock, which consists of a transcriptional-translational negative feedback loop, is regulated by ubiquitination and deubiquitination, two opposite processes that influence the period and robustness of circadian rhythms at the cellular and behavioral levels.