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Ubiquitin proteasome system in circadian rhythm and sleep homeostasis: Lessons from *Drosophila*

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Abstract

Sleep is regulated by two main processes: the circadian clock and sleep homeostasis. Circadian rhythms have been well studied at the molecular level. In the *Drosophila* circadian clock neurons, the core clock proteins are precisely regulated by post-translational modifications and degraded via the ubiquitin-proteasome system (UPS). Sleep homeostasis, however, is less understood; nevertheless, recent reports suggest that proteasome-mediated degradation of core clock proteins or synaptic proteins contributes to the regulation of sleep amount. Here, we review the molecular mechanism of the UPS and summarize the role of protein degradation in the regulation of circadian clock and homeostatic sleep in *Drosophila*. Moreover, we discuss the potential interaction between circadian clock and homeostatic sleep regulation with a prime focus on E3 ubiquitin ligases.

KEYWORDS

circadian clock, circadian rhythm, *Drosophila*, protein homeostasis, sleep, sleep homeostasis, ubiquitin proteasome system

1 | INTRODUCTION

Sleep is a conserved physiological phenomenon in the animal kingdom that is vital for survival, even though it increases the vulnerability to predators. Sleep is necessary for diverse body functions such as metabolic homeostasis (Maguire et al., 2015; Thimgan et al., 2015), learning and memory (Dissel et al., 2015; Gilestro et al., 2009), and clearance of reactive oxidative species (Hill et al., 2018; Vaccaro et al., 2020). Sleep deprivation has been documented to disrupt these functions, leading to premature death (Vaccaro et al., 2020). To ensure the timing and the amount of sleep, sleep is regulated by mainly two processes: circadian clock and sleep homeostasis (Deboer, 2018). Circadian clock regulates behavioral and physiological periodicity, while sleep homeostasis controls the sleep amount in response to the increased demand of sleep following the situations such as sleep

deprivation or sickness, regardless of circadian rhythm (Dubowy & Sehgal, 2017; Toda et al., 2019).

Fruit fly (of the genus *Drosophila*) is a model organism that has been extensively used to investigate the mechanisms of circadian clock at the neural and molecular levels. The circadian rhythm of *Drosophila* is regulated by the transcriptional negative feedback loop of core clock genes including *period* (Konopka & Benzer, 1971), *timeless* (Sehgal et al., 1994), *Clock* (Allada et al., 1998), and *cycle* (Rutila et al., 1998). The core clock genes exhibit molecular oscillations in the clock neurons of the brain, which is the central pacemaker of the circadian rhythms. In mammals, the circadian clock-mediated rest state in the activity rhythm has been comprehended as sleep (Hendricks, Sehgal, et al., 2000). In *Drosophila*, it has been demonstrated that the rest state is a sleep-like state, characterized as behavioral quiescence that lasts for at least 5 min (Hendricks, Finn, et al., 2000; Shaw et al., 2000). During sleep, *Drosophila* exhibits an augmented arousal threshold, making it difficult to respond to external stimuli. However, the sleep state is reversible when the stimuli are stronger than the arousal threshold. After periods of sleep deprivation, fruit flies appear to prolong the duration of their sleep to compensate for the lack of sleep, which is a mechanism of sleep homeostasis. These observations are consistent with the features of sleep homeostasis in mammals (Huber et al., 2004). For the past 20 years, forward genetic screenings using *Drosophila* have identified various genes that regulate sleep. For instance, *Shaker* encodes a subunit of voltage-gated potassium channels and plays a role in electrical excitability, while the mutation of the *Shaker* gene induces a reduction in sleep (Cirelli et al., 2005). Additionally, a mutant of the *redeye* gene encoding the nicotinic acetylcholine receptor α4 shows a similar short sleep phenotype. Redeye protein levels are upregulated in response to the sleep needs and are believed to be a sleep promoting factor under the homeostatic system (Shi et al., 2014). Likewise, a gene that encodes an antimicrobial peptide called *nemuri* has been identified using a gain-of-function screen for sleep induction (Toda et al., 2019). The overexpression of *nemuri* has been reported to prolong the sleep, implying a possible interaction between the immune system and sleep (Toda et al., 2019). Further, Argus, a transmembrane protein which is involved in the autophagosome degradation, is expressed in peptidergic neurons. Notably, *argus* mutants exhibit a short sleep phenotype, indicating the possibility that the degradation of waste products such as proteins in specific neurons could affect sleep (Bedont et al., 2021).

The ubiquitin-proteasome system (UPS) is one of the protein degradation systems, which is highly conserved in eukaryotes and archaea. The UPS is involved in various biological events such as cell cycle progression, cell growth, cell proliferation, endocytosis, elimination of misfolded proteins, and response to environmental stimuli through the degradation of proteins (Nath & Shadan, 2009). The UPS also plays a role in the regulation of circadian clock by degrading core clock proteins (Vriend & Reiter, 2015). In addition, the UPS appears to have the potential to regulate homeostatic sleep in *Drosophila* (Li et al., 2017; Pfeiffenberger & Allada, 2012; Stavropoulos & Young, 2011). Here, we have reviewed the current knowledge of the UPS and its interaction with circadian clock and sleep homeostasis, specifically focusing on the E3 ubiquitin ligases, which have been identified in *Drosophila* (Table 1).

2 | UBIQUITIN PROTEASOME SYSTEM

The mechanism of UPS-mediated protein degradation is divided into two major steps: first, tagging the target protein with ubiquitin, and second, degrading the ubiquitin-tagged proteins via the 26S proteasome. Ubiquitin is a small protein consisting of 76 amino acids that is attached to the target proteins by the catalytic effects of three enzymes namely, the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase enzyme E3 (Bachiller et al., 2020; Ding & Shen, 2008; Leestemaker & Ovaa, 2017). E1 uses ATP to activate ubiquitin with high-energy thioester covalent bonds to the C-terminal glycine of ubiquitin. E2 forms another thioester bond with activated ubiquitin, which is then transferred to E2. E3 catalyzes the transfer of ubiquitin from E2 to the target protein. Subsequently, other activated ubiquitin moieties are added to ubiquitin conjugated target protein, resulting in the synthesizes of a polyubiquitin chain. This chain is then targeted by the 26S proteasome, resulting in target protein degradation (Glickman & Ciechanover, 2002; Leestemaker & Ovaa, 2017).

Approximately 2 E1s, 40 E2s, and more than 600 E3s have been documented to be encoded in the human genome (Leestemaker & Ovaa, 2017; Morreale & Walden, 2016), while there are 8 E1s, 34 E2s, and 207 E3s genes that have been identified in *Drosophila* (Du et al., 2011). Moreover, the E3 enzymes are more diverse than the E1 and E2 enzymes, indicating that E3 ubiquitin ligases determine the specificity of the ubiquitin target proteins (Bachiller et al., 2020; Ding & Shen, 2008). E3 ubiquitin ligases are divided into two major groups: the *R*eally *I*nteresting *N*ew *G*ene (RING) E3, which has a zinc-binding domain called RING or U-box domain; and the *H*omologous to *E*6AP *C*arboxyl *T*erminus (HECT) E3, which has two lobes; one of which interacts with an E2

TABLE 1   Ubiquitin enzymes and ubiquitin-like protein involved in circadian clock and homeostatic sleep regulation

|	Gene	Gene summary	Target	References
Circadian clock	slmb	A substrate receptor of Cullin-RING E3 ligase	Phosphorylated PER	(Chiu et al., 2008; Grima et al., 2002)
	jet	A substrate receptor of Cullin-RING E3 ligase	CRY-bound TIM in light degradation pathway	(Koh et al., 2006; Ozturk et al., 2011; Vaidya et al., 2013)
	Cul3	Cullin scaffold of Cullin-RING E3 ligase	Hypophosphorylated TIM	(Grima et al., 2012)
	ctrip	HECT E3 ligase	CLK	(Lamaze et al., 2011)
Homeostatic sleep regulation	stx	Ubiquitin-like protein	PcG	(Du et al., 2016; Zhao et al., 2021)
	inc	A substrate receptor of Cullin-RING E3 ligase	Unknown	(Pfeiffenberger & Allada, 2012; Pirone et al., 2016; Stavropoulos & Young, 2011)
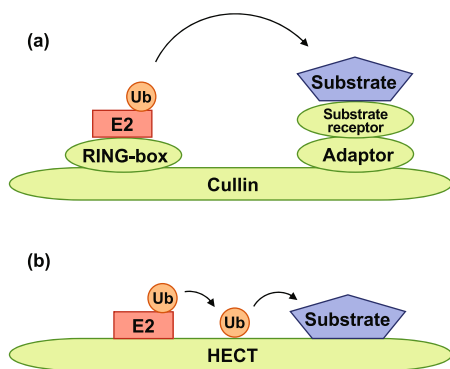
FIGURE 1 Molecular components of E3 ubiquitin ligases. (a) A schematic diagram of Cullin-RING E3 ligases. Cullin-RING E3 ligases (green) directly transfer ubiquitin (orange) from E2 ligase (red) to the substrate (blue). They are composed of multiple subunits (RING-box protein, adaptor protein, and substrate receptor protein), assembled on a Cullin scaffold. The RING-box protein binds to the N-terminus of Cullin, whereas the adaptor protein and substrate receptor protein bind to the C-terminus of Cullin. The RING-box protein binds to the ubiquitin-bound E2, after which ubiquitin is transferred to the substrate bound to the substrate receptor protein. (b) A schematic representation of HECT E3 ligases. The HECT E3 ligases (green) transfer ubiquitin (orange) to the substrate (blue) through a two-step reaction. Ubiquitin is first transferred from E2 ligase (red) to the catalytic site of HECT and subsequently to the substrate. The N-terminal lobe of HECT binds to the ubiquitin-bound E2. The C-terminal lobe of HECT functions as the catalyst for ubiquitin transfer

enzyme while the other interacts with the ubiquitin C terminus (Morreale & Walden, 2016; Rotin & Kumar, 2009). About 95% of E3 ligases belong to RING family (Li et al., 2008).Moreover, the cullin-RING E3 ligases form the largest class of RING family ligases and contribute to approximately 20% of the total ubiquitination in a cell (Petroski & Deshaies, 2005; Soucy et al., 2009). RING E3 catalyzes the direct transfer of ubiquitin from E2 to the target protein (Morreale & Walden, 2016; Figure 1a). In contrast, the HECT family consists of only 28 E3 ligases, constituting about 5% of all the known human E3s (Rotin & Kumar, 2009). Mechanistically, the HECT E3 ligase has been shown to first catalyze the self-transfer of ubiquitin, followed by the transfer of the ubiquitin to the target protein (Morreale & Walden, 2016; Figure 1b).

3 | CIRCADIAN CLOCK AND POST-TRANSLATIONAL MODIFICATION IN *DROSOPHILA*

The circadian rhythms are approximately 24 h cycles, which endogenously regulate the behavior and physiological phenomena in organisms. As the earth takes 24 h to complete one rotation on its axis, the "light and warm period" alternates with the "dark and cold period." Organisms anticipate and adapt to the daily altering environments, and these adaptations are regulated by the clock genes (Dubowy & Sehgal, 2017; Patke et al., 2020). The clock genes are expressed rhythmically, in sync with the environmental changes that affect behavioral and physiological changes throughout a day. The rhythmic expression of clock genes is self-sustained only in the clock cells, also known as circadian pacemakers (Herzog, 2007). The circadian pacemakers regulate the periodical physiology of other cells that do not express the clock genes. For instance, in *Drosophila*, although the lateral horn leucokinin neurons (LHLKs) do not produce the clock proteins, TIMELESS (TIM) and PERIOD (PER), the LHLKs excitability is rhythmically regulated by clock cells, which innervate the LHLKs (Cavey et al., 2016). Therefore, clock cells provide circadian information to other cells called the "clock output cells" and finally induce the behavioral and physiological oscillations.
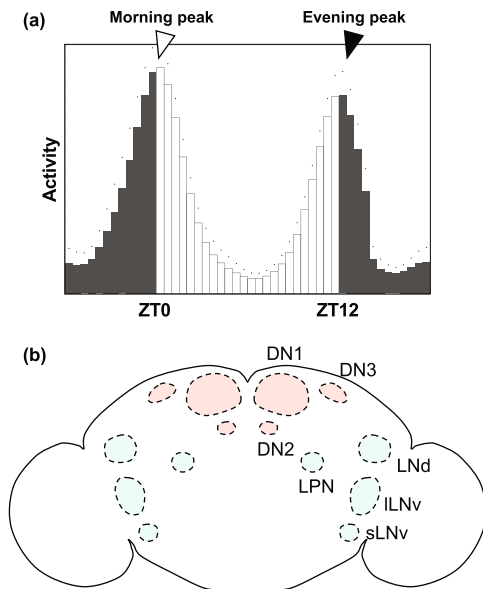
FIGURE 2 Activity rhythm and related neurons in *Drosophila*. (a) Activity of wild-type male *Drosophila* flies in a 12 h:12 h light: dark cycle at 25°C. Light is turned on at Zeitgeber Time (ZT) 0 (morning) turned off at ZT12 (evening). The flies' activity increased during morning and the evening, thus indicating that flies can anticipate the light-on and light-off timings, respectively. (b) The cell body regions of clock neurons in an adult fly's brain. The dorsal neurons (DNs) and lateral neurons (LNs) are shown in red and blue circles, respectively. All LNvs, except for the 5th sLNv, express PDF

Drosophila have been documented to exhibit rhythmic activities. In the laboratory conditions, flies anticipate the transition from dark to light and from light to dark for a 12 h light:12 h dark (LD) cycle, which seems to align with the light/dark transition (Axelrod et al., 2015; Figure 2a). Approximately 150 clock cells, also known as "clock neurons," in the *Drosophila* brain have been demonstrated to regulate this rhythmic activity. The *Drosophila* clock neurons are divided into two groups: lateral neurons, which includes the large and small ventral lateral neurons (lLNvs and sLNvs), dorsal lateral neurons (LNds), lateral posterior neurons (LPNs), and dorsal neurons, including DN1, 2, and 3 (Figure 2b). Nearly all LNvs, except for the 5th sLNvs, express the neuropeptide pigment-dispersing factor (PDF), which is essential for the inter-clock-neuron coordination (Helfrich-Förster, 1997; Kaneko et al., 1997; Rieger et al., 2006). Moreover, the PDF-positive LNvs are required for the morning anticipation of the fly activity (Dubowy & Sehgal, 2017; Renn, Park, et al., 1999). The LNds and PDF-negative LNvs are required for the evening anticipation (Dubowy & Sehgal, 2017; Grima et al., 2004; Stoleru et al., 2004). Likewise, the DN1 play a role in relaying a molecular rhythm, as they receive inputs from the PDF

neurons (Helfrich-Förster, 2003; L. Zhang et al., 2010; Y. Zhang et al., 2010) and send projections to the pars intercerebralis (PI), which is crucial for converting the molecular rhythms into activity rhythms (Cavanaugh et al., 2014).

The well-known clock genes in *Drosophila* are *period* (*per*) and *timeless* (*tim*) (Konopka & Benzer, 1971; Sehgal et al., 1994). These genes are expressed rhythmically, thereby driving the circadian rhythm. PER and TIM proteins have been reported to suppress their own transcription and are degraded during the day. Additionally, the *per* and *tim* mRNA have been documented to be elevated during the day and reach their highest levels in the early evening. In contrast, during the night, the PER and TIM proteins accumulate in the nucleus, which reduces the expression of *per* and *tim* mRNAs. Moreover, the PER and TIM regulate their own transcription through the inhibition of CLOCK (CLK) and CYCLE (CYC) heterodimers, which bind to the enhancer regions of the *per* and *tim* genes. The CLK/CYC heterodimers also bind to the enhancer of clock output genes, which results in the rhythmic and broader physiological changes (Dubowy & Sehgal, 2017; Sharma et al., 2020). Therefore, the cyclic expression of clock genes leads to rhythmic physiological changes.

The CYC protein has been reported to be stabilized by the CLK protein (Liu et al., 2017). Moreover, the *cyc* mRNA expression does not oscillate (Rutila et al., 1998) and the CLK protein is constitutively expressed (Houl et al., 2006); therefore, the CLK/CYC complex persists constantly. To sustain the CLK/CYC-regulated rhythmic expression of *per* and *tim*, the transcriptional capability of CLK/CYC should be accurately regulated by the PER and TIM proteins, which inhibit the CLK/CYC binding to DNA in the nucleus (Lee et al., 1999). Therefore, the entry of PER/TIM into the nucleus is significant and mostly depends on post-translational modifications such as protein phosphorylation. The alpha subunit of casein kinase 2 (CK2α) phosphorylates the Ser149-151-153 of PER and promotes the nuclear entry of PER/TIM (Lin et al., 2002; Lin et al., 2005; Top et al., 2016). While the nuclear entry of PER/TIM is an essential step in inhibiting the expression of clock output genes, the degradation of accumulated PER/TIM is imperative for resuming the CLK/CYC-dependent transcription. It has been reported that PER phosphorylation is the initiation signal for the degradation of PER/TIM. DOUBLE-TIME (DBT), the fly ortholog of mammalian casein kinase 1 (CK1) δ and ε, phosphorylates Ser47 of PER and promotes the degradation of PER (Chiu et al., 2008; Kloss et al., 1998; Price et al., 1998). Additionally, the degradation of PER/TIM after phosphorylation is dependent on the UPS. Therefore, phosphorylation at the differential sites of PER can induce the nuclear entry and degradation of PER/TIM, which serves as the
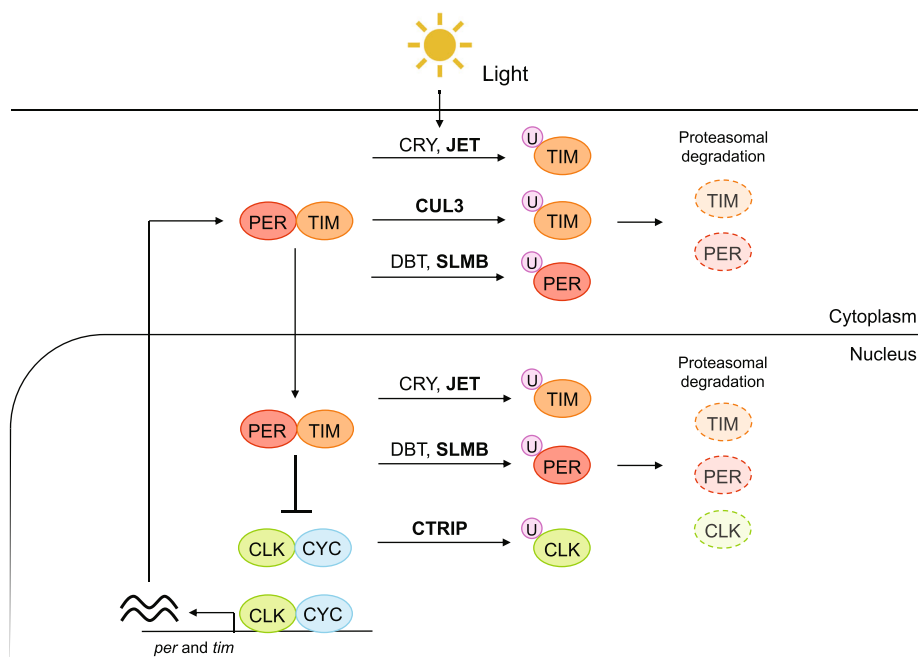
FIGURE 3 E3 ubiquitin ligases and core clock components in *Drosophila*. A scheme of UPS in *Drosophila* circadian clock regulation. When the heterodimer of PER (red) and TIM (orange) is not degraded, the PER/TIM complex accumulates in the nucleus and represses the CLK (green) and CYC (blue) complex. When the PER/TIM is degraded, the CLK/CYC complex promotes the expression of *per* and *tim* (the left side of the scheme). Core clock proteins are degraded by UPS (the right side of the scheme). Light exposure facilitates the binding of CRY to TIM, which allows JET to induce the binding of ubiquitin (pink) to TIM. CUL3 binds to hypophosphorylated TIM and induces ubiquitination of TIM. PER is phosphorylated by DBT in the absence of TIM and subsequently subjected to ubiquitination by SLMB. The transcription factor CLK is ubiquitinated by CTRIP. The ubiquitinated proteins are degraded by 26S proteasome. The boldface shows E3 ligase. The localization of E3 ligases are based on Flybase. It is known that the clock protein is degraded both in cytoplasm and nucleus in mammals (Yoo et al., 2013)

essential mechanism governing the core of the circadian clock.

4 | THE ROLE OF E3 UBIQUITIN LIGASES IN THE *DROSOPHILA* CIRCADIAN CLOCK

The periodic accumulation and degradation of clock proteins largely rely on the UPS-dependent degradation (Stojkovic et al., 2014; Szabó et al., 2018). In the UPS, the specificity of the substrate has been reported to be dependent on the E3 ligases. Here, we describe the E3 ligases that affect the rhythmic behavior and degradation of core clock proteins in *Drosophila*.

Supernumerary limb (*slmb*) gene encodes a Cullin-RING E3 ubiquitin ligase component that functions as a substrate receptor (Figure 1a). Grima et al. have demonstrated that the *slmb* mutant does not show the anticipation of light-off transition under the light/dark (LD) conditions and cannot sustain rhythmic locomotor activity under the constant darkness (DD) conditions (Grima et al., 2002).

Moreover, the Ser47 of PER has been shown to be phosphorylated by DBT, which induces an efficient interaction between PER and SLMB (Chiu et al., 2008). SLMB has been reported to target the phosphorylated PER for proteasomal degradation (Grima et al., 2002; Figure 3). In addition, both the levels of SLMB protein and *slmb* mRNA have been shown to not oscillate (Grima et al., 2002), suggesting that *slmb* is not subjected to a circadian regulation. Additionally, the expression of *slmb* only in PDF-positive LNvs, one of the clock neuron group, can rescue the activity rhythm phenotype of the *slmb* mutant (Grima et al., 2002). Overall, these findings suggest that *slmb* is essential for the regulation of clock gene expression in PDF-positive LNvs, a subset of clock neurons.

JETLAG (*JET*) is also a substrate receptor for the Cullin-RING E3 ubiquitin ligase (Figure 1a). Meanwhile, the *jet* mutants have been reported to show normal activity rhythms under LD, DD, and constant light (LL) conditions (Koh et al., 2006). Under LL conditions, it is known that the wild-type fly shows arrhythmic behavior due to the constant degradation of TIM by light. The degradation of TIM requires light and CRY, the blue light photoreceptor

cryptochrome (Ceriani et al., 1999). Studies have shown that light induces conformational changes in CRY, which then binds to TIM, allowing the JET protein to bind to TIM to induce ubiquitin-dependent TIM degradation (Lamba et al., 2014; Ozturk et al., 2011; Vaidya et al., 2013; Figure 3). Additionally, the JET protein contains leucine-rich repeats (LRRs), which is a protein–protein interaction domain (Koh et al., 2006). Therefore, it is speculated that the LRRs of JET bind to the CRY-bound TIM.

CULLIN-3 (CUL3), a member of the CULLIN family, is a scaffold of the Cullin-RING E3 ubiquitin ligase involved in the function of the circadian clock (Figure 1a). It has been reported that the knockdown of *Cul3* E3 in a limited number of clock neurons induces defects in morning anticipation under LD conditions and behavioral arrhythmicity in DD conditions, whereas the knockdown of *Cul3* in all clock neurons results in 100% lethality (Grima et al., 2012). Likewise, CUL3 binds to hypophosphorylated TIM and controls the TIM oscillation (Grima et al., 2012; Guo et al., 2014); however, it is unclear which substrate receptor protein binds to CUL3 and bears the specificity for TIM (Figure 3).

Besides the Cullin-RING E3 ubiquitin ligase, the HECT E3 ubiquitin ligase, *circadian trip* (*ctrip*), has also been reported as a clock control gene (Lamaze et al., 2011; Figure 1b). CTRIP has been shown to contain a WWE protein–protein interaction domain, which is a putative binding site for the target protein, CLK. The expression of *ctrip* is strictly restricted to the PDF-positive LNvs (Lamaze et al., 2011), indicating that *ctrip* contributes to the degradation of the core clock gene (Figure 3). Additionally, the knockdown of *ctrip* leads to high levels of CLK protein at all times and extends the period length of endogenous rhythm in the absence of external stimuli, such as light (Lamaze et al., 2011).

Together, the E3 ubiquitin ligases, which bind to clock proteins, affect the behavior and molecular cycling associated with circadian clock. Notably, the E3 ligases mentioned above do not exhibit oscillation in their expression, suggesting that ubiquitin-dependent degradation is regulated by other systems such as protein phosphorylation, which are important for the precise cycling of clock proteins (Stojkovic et al., 2014). However, the absence of E3 ligases does not result in the abolishment of the core clock protein functions. Therefore, the E3 ligases play an essential role in the degradation of core clock proteins.

5 | PROTEASOMAL DEGRADATION IN *DROSOPHILA* SLEEP HOMEOSTASIS

Circadian clock induces sleep after the beginning of the dark period, and to wake up in advance of the light period, whereas the homeostatic regulation of sleep determines the amount of sleep to meet the body's sleep requirements. Sleep homeostasis in *Drosophila* shares the features of mammalian sleep homeostasis, with the intensity and duration of sleep rebound being dependent on the prior awakening time or sleep deprivation (Huber et al., 2004). When sleep-deprived, the duration of sleep in flies is less fragmented and is accompanied by high arousal thresholds (Figure 4a). Moreover, the sleep homeostatic system depends on the R5 neurons (previously referred to as R2 neurons) in the ellipsoid body (EB), which is a doughnut-shaped synaptic neuropil domain in the adult fly brain. The R5 neurons have been reported to activate the dorsal fan-shaped body (dFB), thus integrating various sleep signals to promote sleep (Dubowy & Sehgal, 2017; Figure 4b). Moreover, the activation of the R5 neurons has been shown to strongly induce sleep (Liu et al., 2016). Slow-wave oscillation, which is characteristic of deep sleep in the vertebrate brain and is believed to be the synchronization of neural activity, has been discovered in R5 neurons (Raccuglia et al., 2019). Therefore, while the homeostatic regulation of sleep has been revealed in neural circuits, it is poorly understood at the molecular level. For instance, it is not known what molecule is specifically responsible for inducing the homeostatic sleep drive.

One of the vital functions of sleep is the clearance of inessential byproducts from the brain. In the mammalian brain, sleep modulates interstitial space size and induces cerebrospinal fluid influx, resulting in the clearance of β-amyloids, a toxic metabolic byproduct, from the brain (Xie et al., 2013). Aggregation of such degradation byproducts leads to neural injury and ultimately the Alzheimer's disease (Mattson, 1994). Moreover, Varshavsky hypothesized that the fragmented proteins cleaved by proteases accumulate when the organism is awake and are degraded by the proteasome system during the night (Varshavsky, 2012, 2019b). Additionally, the calpains and caspases are present at post-synaptic densities (PSDs) and cleave post-synaptic proteins (Varshavsky, 2019b). The resultant cleaved proteins generate two fragments: a protein containing a new N-terminus and a protein containing a new C-terminus (Varshavsky, 2019b). The new N-terminus and C-terminus function as degrons, which are the targets of ubiquitin-dependent degradation (Varshavsky, 2019a). In summary, the UPS is likely required to degrade the accumulated protein fragments during sleep. Therefore, we have hereby deliberated on two proteasome-related molecules that have the potential to reveal the regulatory mechanism of sleep homeostasis at the molecular level in *Drosophila*.

Forward genetic screens have identified *insomniac* (*inc*), which a mutant exhibiting a significant reduction in sleep (Stavropoulos & Young, 2011). Moreover, *inc* is an ortholog of the potassium channel tetramerization
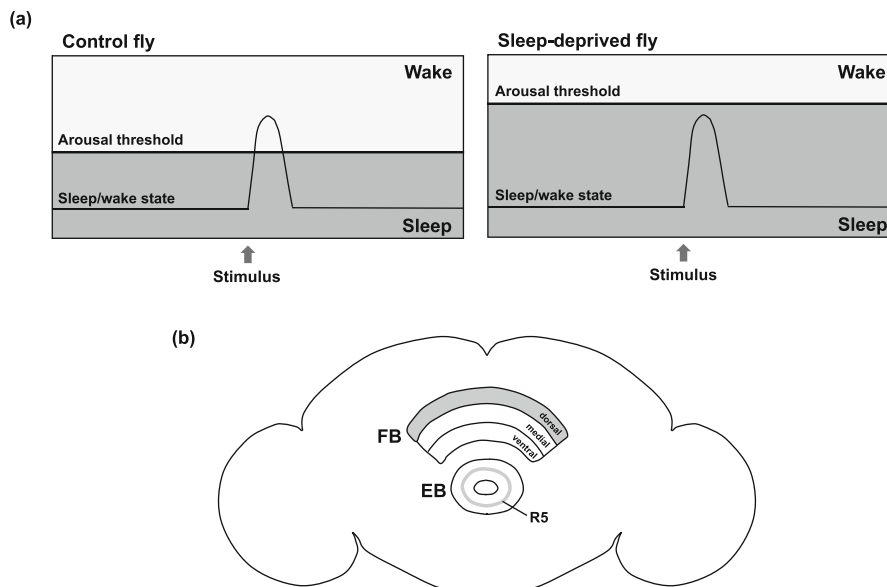
FIGURE 4 Sleep homeostasis and related neurons in *Drosophila*. (a) Schematics showing arousal threshold and sleep/wake states. A control fly is aroused when subjected to a stimulus that exceeds the arousal threshold (left panel). Moreover, the arousal threshold is higher in a sleep-deprived fly than in the control fly. Therefore, the sleep-deprived fly is not aroused even when subjected to intense stimuli (right panel). (b) A diagram showing the regions in the adult fly brain involved in the homeostatic sleep regulation. The fan-shaped body (FB) comprises three domains: a dorsal domain, a medial domain, and a ventral domain. Only the dorsal domain of FB (dFB) regulates sleep by the activation of R5 neurons, a type of R neurons arborizing as a ring in the ellipsoid body (EB) (Omoto et al., 2017; Renn, Armstrong, et al., 1999), thereby driving the regulation of sleep homeostasis

domain (KCTD) subfamily in vertebrates (Stavropoulos & Young, 2011). Several KCTD subfamilies function as substrate receptors for CUL3 (Cho et al., 2020; He et al., 2018; Rutz et al., 2015; Stavropoulos & Young, 2011). Therefore, INC is a component of SCF E3 ligase that might be involved in the induction of proteasomal degradation. Moreover, *Cul3* knockdown reduces sleep, suggesting that the INC/CUL3 complex plays a vital role in regulating sleep and maintaining the sleep homeostatic system (Pfeiffenberger & Allada, 2012; Pirone et al., 2016; Stavropoulos & Young, 2011). The *inc* gene is expressed in sleep regulatory neurons, including EB, FB, and mushroom bodies (MBs); the INC protein is localized in the cytosol, or the pre- and post-synaptic compartments of neurons (Li et al., 2017). These findings imply that the INC/CUL3 complex regulates sleep by degrading its targets that are localized at the synapse. According to the hypothesis that the accumulated protein fragments are vital for the sleep/wake cycle, the INC/CUL3 complex may potentially be involved in the degradation of the fragments derived from synapse proteins cleaved by proteases. Protein structure analysis and in vitro isothermal calorimetry revealed that *Drosophila* Golgi reassembly stacking protein (dGRASP) is thought to be a potential target of INC/CUL3 complex (Pirone et al., 2016). However, the genetic interaction between INC and dGRASP in vivo requires further study. Therefore, the identification of the

INC/CUL3 complex target may lead to the elucidation of the molecular regulation mechanism of sleep homeostasis.

The ubiquitin-like protein *Stuxnet* (*stx*) promotes the degradation of an epigenetic repressor polycomb group (PcG) by the proteasome, independently of ubiquitination (Du et al., 2016). The *stx* mutant increases sleep duration and reduces sleep latency, indicating that sleep pressure is elevated in the mutants (Zhao et al., 2021). Stx protein is localized in the EB, which is an important region for homeostatic sleep regulation. Moreover, it has been reported to be downregulated in response to octopamine and reduces the expression of the sleep-inhibiting octopamine β2 receptor through epigenetic regulation in EB (Zhao et al., 2021).

6 | CONCLUSION AND FUTURE PERSPECTIVE

Sleep behavior is poorly understood at the molecular level. Contrary to the neural circuit of sleep regulation, the molecular agents responsible for sleep regulation have not been well elucidated. The INC/CUL3 complex is predicted to play a role in proteasomal degradation (Cho et al., 2020; He et al., 2018; Li et al., 2017; Pirone et al., 2016; Rutz et al., 2015; Stavropoulos & Young,

2011) and the substrate degraded by INC/CUL3-dependent UPS may be a key factor in regulating the sleep. Thus, the INC/CUL3 complex is considered to be significant in elucidating the molecular mechanisms underlying sleep regulation.

CUL3 plays crucial role in the regulation of circadian clock through the degradation of core clock protein, TIM (Grima et al., 2012), and the homeostatic sleep regulation (Stavropoulos & Young, 2011). Moreover, CUL3 in clock neurons is essential for TIM degradation in the circadian clock, whereas the CUL3/INC complex in sleep regulatory neurons is important for unknown-target degradation (Pfeiffenberger & Allada, 2012; Stavropoulos & Young, 2011). Besides, INC is localized in the PDF-positive neurons (Li et al., 2017). The substrate receptor protein of CUL3 for degradation of TIM has not been reported yet. However, the difference in the substrate receptor protein of CUL3 possibly contributes to both the circadian clock and homeostatic sleep regulation (Freeman et al., 2013). Consequently, identifying the substrate receptor protein of CUL3 within the circadian clock may be useful in elucidating the interaction between the circadian clock and homeostatic sleep at the molecular level.

It is hypothesized that synaptic properties contribute significantly to the regulation and function of sleep. For instance, sleep loss induces an increased accumulation of the synaptic proteins (Gilestro et al., 2009) in the presynaptic active zone, which is responsible for the occurrence of high sleep pressure in *Drosophila* (Huang et al., 2020). These facts imply that the elevated levels of synaptic proteins induce sleep to eliminate the accumulated synaptic protein, which may be one of the primary functions of sleep. Varshavsky hypothesized that another function of sleep is to eliminate the fragmented proteins at the synapse accumulated while awake (Varshavsky, 2012, 2019b). According to this hypothesis, fragmented proteins are eliminated by the UPS. In summary, the UPS is predicted to contribute to the function of sleep through the degradation of synaptic proteins. Moreover, the localization of INC in pre- and post-synaptic compartments (Li et al., 2017) implies a potential relationship between sleep and the degradation of synaptic proteins.

CONFLICT OF INTEREST
The authors declare no potential conflict of interests.

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REFERENCES
Allada, R., White, N. E., So, W. V., Hall, J. C., & Rosbash, M. (1998). A mutant Drosophila homolog of mammalian clock disrupts circadian rhythms and transcription of period and timeless. *Cell*, *93*(5), 791–804. https://doi.org/10.1016/s0092-8674(00)81440-3

Axelrod, S., Saez, L., & Young, M. W. (2015). Studying circadian rhythm and sleep using genetic screens in Drosophila. *Methods in Enzymology*, *551*, 3–27. https://doi.org/10.1016/bs.mie.2014.10.026

Bachiller, S., Alonso-Bellido, I. M., Real, L. M., Pérez-Villegas, E. M., Venero, J. L., Deierborg, T., Armengol, J. Á., & Ruiz, R. (2020). The ubiquitin proteasome system in neuromuscular disorders: Moving beyond movement. *International Journal of Molecular Sciences*, *21*(17), 6429. https://doi.org/10.3390/ijms21176429

Bedont, J. L., Toda, H., Shi, M., Park, C. H., Quake, C., Stein, C., Kolesnik, A., & Sehgal, A. (2021). Short and long sleeping mutants reveal links between sleep and macroautophagy. *eLife*, *10*, e64140. https://doi.org/10.7554/eLife.64140

Cavanaugh, D. J., Geratowski, J. D., Wooltorton, J. R., Spaethling, J. M., Hector, C. E., Zheng, X., Johnson, E. C., Eberwine, J. H., & Sehgal, A. (2014). Identification of a circadian output circuit for rest: Activity rhythms in Drosophila. *Cell*, *157*(3), 689–701. https://doi.org/10.1016/j.cell.2014.02.024

Cavey, M., Collins, B., Bertet, C., & Blau, J. (2016). Circadian rhythms in neuronal activity propagate through output circuits. *Nature Neuroscience*, *19*(4), 587–595. https://doi.org/10.1038/nn.4263

Ceriani, M. F., Darlington, T. K., Staknis, D., Más, P., Petti, A. A., Weitz, C. J., & Kay, S. A. (1999). Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science (New York, N.Y.)*, *285*(5427), 553–556. https://doi.org/10.1126/science.285.5427.553

Chiu, J. C., Vanselow, J. T., Kramer, A., & Edery, I. (2008). The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. *Genes & Development*, *22*(13), 1758–1772. https://doi.org/10.1101/gad.1682708

Cho, H. J., Ryu, K. J., Baek, K. E., Lim, J., Kim, T., Song, C. Y., Yoo, J., & Lee, H. G. (2020). Cullin 3/KCTD5 promotes the ubiquitination of rho guanine nucleotide dissociation inhibitor 1 and regulates its stability. *Journal of Microbiology and Biotechnology*, *30*(10), 1488–1494. https://doi.org/10.4014/jmb.2007.07033

Cirelli, C., Bushey, D., Hill, S., Huber, R., Kreber, R., Ganetzky, B., & Tononi, G. (2005). Reduced sleep in Drosophila Shaker mutants. *Nature*, *434*(7037), 1087–1092. https://doi.org/10.1038/nature03486

Deboer, T. (2018). Sleep homeostasis and the circadian clock: Do the circadian pacemaker and the sleep homeostat influence each other's functioning? *Neurobiology of Sleep and Circadian Rhythms*, *5*, 68–77. https://doi.org/10.1016/j.nbscr.2018.02.003

Ding, M., & Shen, K. (2008). The role of the ubiquitin proteasome system in synapse remodeling and neurodegenerative diseases. *BioEssays*, *30*(11–12), 1075–1083. https://doi.org/10.1002/bies.20843

Dissel, S., Melnattur, K., & Shaw, P. J. (2015). Sleep, performance, and memory in flies. *Current Sleep Medicine Reports*, *1*(1), 47–54. https://doi.org/10.1007/s40675-014-0006-4

Du, J., Zhang, J., He, T., Li, Y., Su, Y., Tie, F., Liu, M., Harte, P. J., & Zhu, A. J. (2016). Stuxnet facilitates the degradation of polycomb protein during development. *Developmental Cell*, *37*(6), 507–519. https://doi.org/10.1016/j.devcel.2016.05.013

Du, J., Zhang, J., Su, Y., Liu, M., Ospina, J. K., Yang, S., & Zhu, A. J. (2011). In vivo RNAi screen reveals neddylation genes as novel regulators of hedgehog signaling. *PLoS One*, *6*(9), e24168. https://doi.org/10.1371/journal.pone.0024168

Dubowy, C., & Sehgal, A. (2017). Circadian rhythms and sleep in *Drosophila melanogaster*. *Genetics*, *205*(4), 1373–1397. https://doi.org/10.1534/genetics.115.185157

Freeman, A. A., Mandilaras, K., Missirlis, F., & Sanyal, S. (2013). An emerging role for Cullin-3 mediated ubiquitination in sleep and circadian rhythm: Insights from *Drosophila*. *Fly*, *7*(1), 39–43. https://doi.org/10.4161/fly.23506

Gilestro, G. F., Tononi, G., & Cirelli, C. (2009). Widespread changes in synaptic markers as a function of sleep and wakefulness in *Drosophila*. *Science (New York, N.Y.)*, *324*(5923), 109–112. https://doi.org/10.1126/science.1166673

Glickman, M. H., & Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. *Physiological Reviews*, *82*(2), 373–428. https://doi.org/10.1152/physrev.00027.2001

Grima, B., Chélot, E., Xia, R., & Rouyer, F. (2004). Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. *Nature*, *431*(7010), 869–873. https://doi.org/10.1038/nature02935

Grima, B., Dognon, A., Lamouroux, A., Chélot, E., & Rouyer, F. (2012). CULLIN-3 controls TIMELESS oscillations in the *Drosophila* circadian clock. *PLoS Biology*, *10*(8), e1001367. https://doi.org/10.1371/journal.pbio.1001367

Grima, B., Lamouroux, A., Chélot, E., Papin, C., Limbourg-Bouchon, B., & Rouyer, F. (2002). The F-box protein slimb controls the levels of clock proteins period and timeless. *Nature*, *420*(6912), 178–182. https://doi.org/10.1038/nature01122

Guo, F., Cerullo, I., Chen, X., & Rosbash, M. (2014). PDF neuron firing phase-shifts key circadian activity neurons in *Drosophila*. *eLife*, *3*, e02780. https://doi.org/10.7554/eLife.02780

He, H., Peng, Y., Fan, S., Chen, Y., Zheng, X., & Li, C. (2018). Cullin3/KCTD5 induces monoubiquitination of ΔNp63α and impairs its activity. *FEBS Letters*, *592*(13), 2334–2340. https://doi.org/10.1002/1873-3468.13104

Helfrich-Förster, C. (1997). Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila* melanogaster. *The Journal of Comparative Neurology*, *380*(3), 335–354. https://doi.org/10.1002/(sici)1096-9861(19970414)380:3<335::aid-cne4>3.0.co;2-3

Helfrich-Förster, C. (2003). The neuroarchitecture of the circadian clock in the brain of *Drosophila* melanogaster. *Microscopy Research and Technique*, *62*(2), 94–102. https://doi.org/10.1002/jemt.10357

Hendricks, J. C., Finn, S. M., Panckeri, K. A., Chavkin, J., Williams, J. A., Sehgal, A., & Pack, A. I. (2000). Rest in *Drosophila* is a sleep-like state. *Neuron*, *25*(1), 129–138. https://doi.org/10.1016/s0896-6273(00)80877-6

Hendricks, J. C., Sehgal, A., & Pack, A. I. (2000). The need for a simple animal model to understand sleep. *Progress in Neurobiology*, *61*(4), 339–351. https://doi.org/10.1016/s0301-0082(99)00048-9

Herzog, E. D. (2007). Neurons and networks in daily rhythms. *Nature Reviews. Neuroscience*, *8*(10), 790–802. https://doi.org/10.1038/nrn2215

Hill, V. M., O'Connor, R. M., Sissoko, G. B., Irobunda, I. S., Leong, S., Canman, J. C., Stavropoulos, N., & Shirasu-Hiza, M. (2018). A bidirectional relationship between sleep and oxidative stress in *Drosophila*. *PLoS Biology*, *16*(7), e2005206. https://doi.org/10.1371/journal.pbio.2005206

Houl, J. H., Yu, W., Dudek, S. M., & Hardin, P. E. (2006). *Drosophila* CLOCK is constitutively expressed in circadian oscillator and non-oscillator cells. *Journal of Biological Rhythms*, *21*(2), 93–103. https://doi.org/10.1177/0748730405283697

Huang, S., Piao, C., Beuschel, C. B., Götz, T., & Sigrist, S. J. (2020). Presynaptic active zone plasticity encodes sleep need in *Drosophila*. *Current Biology*, *30*(6), 1077–1091.e5. https://doi.org/10.1016/j.cub.2020.01.019

Huber, R., Hill, S. L., Holladay, C., Biesiadecki, M., Tononi, G., & Cirelli, C. (2004). Sleep homeostasis in *Drosophila* melanogaster. *Sleep*, *27*(4), 628–639. https://doi.org/10.1093/sleep/27.4.628

Kaneko, M., Helfrich-Förster, C., & Hall, J. C. (1997). Spatial and temporal expression of the period and timeless genes in the developing nervous system of *Drosophila*: Newly identified pacemaker candidates and novel features of clock gene product cycling. *The Journal of Neuroscience*, *17*(17), 6745–6760. https://doi.org/10.1523/JNEUROSCI.17-17-06745.1997

Kloss, B., Price, J. L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C. S., & Young, M. W. (1998). The *Drosophila* clock gene double-time encodes a protein closely related to human casein kinase Iε. *Cell*, *94*(1), 97–107. https://doi.org/10.1016/s0092-8674(00)81225-8

Koh, K., Zheng, X., & Sehgal, A. (2006). JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. *Science (New York, N.Y.)*, *312*(5781), 1809–1812. https://doi.org/10.1126/science.1124951

Konopka, R. J., & Benzer, S. (1971). Clock mutants of *Drosophila* melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *68*(9), 2112–2116. https://doi.org/10.1073/pnas.68.9.2112

Lamaze, A., Lamouroux, A., Vias, C., Hung, H. C., Weber, F., & Rouyer, F. (2011). The E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in *Drosophila*. *EMBO Reports*, *12*(6), 549–557. https://doi.org/10.1038/embor.2011.64

Lamba, P., Bilodeau-Wentworth, D., Emery, P., & Zhang, Y. (2014). Morning and evening oscillators cooperate to reset circadian behavior in response to light input. *Cell Reports*, *7*(3), 601–608. https://doi.org/10.1016/j.celrep.2014.03.044

Lee, C., Bae, K., & Edery, I. (1999). PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: A basis for circadian transcription. *Molecular and Cellular Biology*, 19(8), 5316–5325. https://doi.org/10.1128/MCB.19.8.5316

Leestemaker, Y., & Ovaa, H. (2017). Tools to investigate the ubiquitin proteasome system. *Drug Discovery Today: Technologies*, 26, 25–31. https://doi.org/10.1016/j.ddtec.2017.11.006

Li, Q., Kellner, D. A., Hatch, H., Yumita, T., Sanchez, S., Machold, R. P., Frank, C. A., & Stavropoulos, N. (2017). Conserved properties of *Drosophila* insomniac link sleep regulation and synaptic function. *PLoS Genetics*, 13(5), e1006815. https://doi.org/10.1371/journal.pgen.1006815

Li, W., Bengtson, M. H., Ulbrich, A., Matsuda, A., Reddy, V. A., Orth, A., Chanda, S. K., Batalov, S., & Joazeiro, C. A. (2008). Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS One*, 3(1), e1487. https://doi.org/10.1371/journal.pone.0001487

Lin, J. M., Kilman, V. L., Keegan, K., Paddock, B., Emery-Le, M., Rosbash, M., & Allada, R. (2002). A role for casein kinase 2alpha in the *Drosophila* circadian clock. *Nature*, 420(6917), 816–820. https://doi.org/10.1038/nature01235

Lin, J. M., Schroeder, A., & Allada, R. (2005). In vivo circadian function of casein kinase 2 phosphorylation sites in *Drosophila* period. *The Journal of Neuroscience*, 25(48), 11175–11183. https://doi.org/10.1523/JNEUROSCI.2159-05.2005

Liu, S., Liu, Q., Tabuchi, M., & Wu, M. N. (2016). Sleep drive is encoded by neural plastic changes in a dedicated circuit. *Cell*, 165(6), 1347–1360. https://doi.org/10.1016/j.cell.2016.04.013

Liu, T., Mahesh, G., Yu, W., & Hardin, P. E. (2017). CLOCK stabilizes CYCLE to initiate clock function in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 114(41), 10972–10977. https://doi.org/10.1073/pnas.1707143114

Maguire, S. E., Rhoades, S., Chen, W. F., Sengupta, A., Yue, Z., Lim, J. C., Mitchell, C. H., Weljie, A. M., & Sehgal, A. (2015). Independent effects of γ-aminobutyric acid transaminase (GABAT) on metabolic and sleep homeostasis. *The Journal of Biological Chemistry*, 290(33), 20407–20416. https://doi.org/10.1074/jbc.M114.602276

Mattson, M. P. (1994). Calcium and neuronal injury in Alzheimer's disease. Contributions of beta-amyloid precursor protein mismetabolism, free radicals, and metabolic compromise. *Annals of the New York Academy of Sciences*, 747, 50–76.

Morreale, F. E., & Walden, H. (2016). Types of ubiquitin ligases. *Cell*, 165(1), 248–248.e1. https://doi.org/10.1016/j.cell.2016.03.003

Nath, D., & Shadan, S. (2009). The ubiquitin system. *Nature*, 458(7237), 421. https://doi.org/10.1038/458421a

Omoto, J. J., Keleş, M. F., Nguyen, B. M., Bolanos, C., Lovick, J. K., Frye, M. A., & Hartenstein, V. (2017). Visual input to the *Drosophila* central complex by developmentally and functionally distinct neuronal populations. *Current Biology*, 27(8), 1098–1110. https://doi.org/10.1016/j.cub.2017.02.063

Ozturk, N., Selby, C. P., Annayev, Y., Zhong, D., & Sancar, A. (2011). Reaction mechanism of *Drosophila* cryptochrome. *Proceedings of the National Academy of Sciences of the United States of America*, 108(2), 516–521. https://doi.org/10.1073/pnas.1017093108

Patke, A., Young, M. W., & Axelrod, S. (2020). Molecular mechanisms and physiological importance of circadian rhythms. *Nature Reviews. Molecular Cell Biology*, 21(2), 67–84. https://doi.org/10.1038/s41580-019-0179-2

Petroski, M. D., & Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nature Reviews. Molecular Cell Biology*, 6(1), 9–20. https://doi.org/10.1038/nrm1547

Pfeiffenberger, C., & Allada, R. (2012). Cul3 and the BTB adaptor insomniac are key regulators of sleep homeostasis and a dopamine arousal pathway in *Drosophila*. *PLoS Genetics*, 8(10), e1003003. https://doi.org/10.1371/journal.pgen.1003003

Pirone, L., Smaldone, G., Esposito, C., Balasco, N., Petoukhov, M. V., Spilotros, A., Svergun, D. I., Di Gaetano, S., Vitagliano, L., & Pedone, E. M. (2016). Proteins involved in sleep homeostasis: Biophysical characterization of INC and its partners. *Biochimie*, 131, 106–114. https://doi.org/10.1016/j.biochi.2016.09.013

Price, J. L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., & Young, M. W. (1998). Double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell*, 94(1), 83–95. https://doi.org/10.1016/s0092-8674(00)81224-6

Raccuglia, D., Huang, S., Ender, A., Heim, M. M., Laber, D., Suárez-Grimalt, R., Liotta, A., Sigrist, S. J., Geiger, J., & Owald, D. (2019). Network-specific synchronization of electrical slow-wave oscillations regulates sleep drive in *Drosophila*. *Current Biology*, 29(21), 3611–3621. https://doi.org/10.1016/j.cub.2019.08.070

Renn, S. C., Armstrong, J. D., Yang, M., Wang, Z., An, X., Kaiser, K., & Taghert, P. H. (1999). Genetic analysis of the *Drosophila* ellipsoid body neuropil: Organization and development of the central complex. *Journal of Neurobiology*, 41(2), 189–207.

Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C., & Taghert, P. H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell*, 99(7), 791–802. https://doi.org/10.1016/s0092-8674(00)81676-1

Rieger, D., Shafer, O. T., Tomioka, K., & Helfrich-Förster, C. (2006). Functional analysis of circadian pacemaker neurons in *Drosophila* melanogaster. *The Journal of Neuroscience*, 26(9), 2531–2543. https://doi.org/10.1523/JNEUROSCI.1234-05.2006

Rotin, D., & Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. *Nature Reviews. Molecular Cell Biology*, 10(6), 398–409. https://doi.org/10.1038/nrm2690

Rutila, J. E., Suri, V., Le, M., So, W. V., Rosbash, M., & Hall, J. C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila* period and timeless. *Cell*, 93(5), 805–814. https://doi.org/10.1016/s0092-8674(00)81441-5

Rutz, N., Heilbronn, R., & Weger, S. (2015). Interactions of cullin3/KCTD5 complexes with both cytoplasmic and nuclear proteins: Evidence for a role in protein stabilization. *Biochemical and Biophysical Research Communications*, 464(3), 922–928. https://doi.org/10.1016/j.bbrc.2015.07.069

Sehgal, A., Price, J. L., Man, B., & Young, M. W. (1994). Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant timeless. *Science (New York, N.Y.)*, 263(5153), 1603–1606. https://doi.org/10.1126/science.8128246

Sharma, A., Lee, S., Kim, H., Yoon, H., Ha, S., & Kang, S. U. (2020). Molecular crosstalk between circadian rhythmicity and the

development of neurodegenerative disorders. *Frontiers in Neuroscience*, *14*, 844. https://doi.org/10.3389/fnins.2020.00844

Shaw, P. J., Cirelli, C., Greenspan, R. J., & Tononi, G. (2000). Correlates of sleep and waking in *Drosophila* melanogaster. *Science (New York, N.Y.)*, *287*(5459), 1834–1837. https://doi.org/10.1126/science.287.5459.1834

Shi, M., Yue, Z., Kuryatov, A., Lindstrom, J. M., & Sehgal, A. (2014). Identification of Redeye, a new sleep-regulating protein whose expression is modulated by sleep amount. *eLife*, *3*, e01473. https://doi.org/10.7554/eLife.01473

Soucy, T. A., Smith, P. G., Milhollen, M. A., Berger, A. J., Gavin, J. M., Adhikari, S., Brownell, J. E., Burke, K. E., Cardin, D. P., Critchley, S., Cullis, C. A., Doucette, A., Garnsey, J. J., Gaulin, J. L., Gershman, R. E., Lublinsky, A. R., McDonald, A., Mizutani, H., Narayanan, U., ... Langston, S. P. (2009). An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature*, *458*(7239), 732–736. https://doi.org/10.1038/nature07884

Stavropoulos, N., & Young, M. W. (2011). Insomniac and Cullin-3 regulate sleep and wakefulness in *Drosophila*. *Neuron*, *72*(6), 964–976. https://doi.org/10.1016/j.neuron.2011.12.003

Stojkovic, K., Wing, S. S., & Cermakian, N. (2014). A central role for ubiquitination within a circadian clock protein modification code. *Frontiers in Molecular Neuroscience*, *7*, 69. https://doi.org/10.3389/fnmol.2014.00069

Stoleru, D., Peng, Y., Agosto, J., & Rosbash, M. (2004). Coupled oscillators control morning and evening locomotor behaviour of *Drosophila*. *Nature*, *431*(7010), 862–868. https://doi.org/10.1038/nature02926

Szabó, Á., Papin, C., Cornu, D., Chélot, E., Lipinszki, Z., Udvardy, A., Redeker, V., Mayor, U., & Rouyer, F. (2018). Ubiquitylation dynamics of the clock cell proteome and TIMELESS during a circadian cycle. *Cell Reports*, *23*(8), 2273–2282. https://doi.org/10.1016/j.celrep.2018.04.064

Thimgan, M. S., Seugnet, L., Turk, J., & Shaw, P. J. (2015). Identification of genes associated with resilience/vulnerability to sleep deprivation and starvation in *Drosophila*. *Sleep*, *38*(5), 801–814. https://doi.org/10.5665/sleep.4680

Toda, H., Williams, J. A., Gulledge, M., & Sehgal, A. (2019). A sleep-inducing gene, *nemuri*, links sleep and immune function in *Drosophila*. *Science (New York, N.Y.)*, *363*(6426), 509–515. https://doi.org/10.1126/science.aat1650

Top, D., Harms, E., Syed, S., Adams, E. L., & Saez, L. (2016). GSK-3 and CK2 kinases converge on timeless to regulate the master clock. *Cell Reports*, *16*(2), 357–367. https://doi.org/10.1016/j.celrep.2016.06.005

Vaccaro, A., Kaplan Dor, Y., Nambara, K., Pollina, E. A., Lin, C., Greenberg, M. E., & Rogulja, D. (2020). Sleep loss can cause death through accumulation of reactive oxygen species in the gut. *Cell*, *181*(6), 1307–1328.e15. https://doi.org/10.1016/j.cell.2020.04.049

Vaidya, A. T., Top, D., Manahan, C. C., Tokuda, J. M., Zhang, S., Pollack, L., Young, M. W., & Crane, B. R. (2013). Flavin reduction activates *Drosophila* cryptochrome. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(51), 20455–20460. https://doi.org/10.1073/pnas.1313336110

Varshavsky, A. (2012). Augmented generation of protein fragments during wakefulness as the molecular cause of sleep: A hypothesis. *Protein Science*, *21*(11), 1634–1661. https://doi.org/10.1002/pro.2148

Varshavsky, A. (2019a). N-degron and C-degron pathways of protein degradation. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(2), 358–366. https://doi.org/10.1073/pnas.1816596116

Varshavsky, A. (2019b). On the cause of sleep: Protein fragments, the concept of sentinels, and links to epilepsy. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(22), 10773–10782. https://doi.org/10.1073/pnas.1904709116

Vriend, J., & Reiter, R. J. (2015). Melatonin feedback on clock genes: A theory involving the proteasome. *Journal of Pineal Research*, *58*(1), 1–11. https://doi.org/10.1111/jpi.12189

Xie, L., Kang, H., Xu, Q., Chen, M. J., Liao, Y., Thiyagarajan, M., O'Donnell, J., Christensen, D. J., Nicholson, C., Iliff, J. J., Takano, T., Deane, R., & Nedergaard, M. (2013). Sleep drives metabolite clearance from the adult brain. *Science (New York, N.Y.)*, *342*(6156), 373–377. https://doi.org/10.1126/science.1241224

Yoo, S. H., Mohawk, J. A., Siepka, S. M., Shan, Y., Huh, S. K., Hong, H. K., Kornblum, I., Kumar, V., Koike, N., Xu, M., Nussbaum, J., Liu, X., Chen, Z., Chen, Z. J., Green, C. B., & Takahashi, J. S. (2013). Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm. *Cell*, *152*(5), 1091–1105. https://doi.org/10.1016/j.cell.2013.01.055

Zhang, L., Chung, B. Y., Lear, B. C., Kilman, V. L., Liu, Y., Mahesh, G., Meissner, R. A., Hardin, P. E., & Allada, R. (2010). DN1(p) circadian neurons coordinate acute light and PDF inputs to produce robust daily behavior in *Drosophila*. *Current Biology*, *20*(7), 591–599. https://doi.org/10.1016/j.cub.2010.02.056

Zhang, Y., Liu, Y., Bilodeau-Wentworth, D., Hardin, P. E., & Emery, P. (2010). Light and temperature control the contribution of specific DN1 neurons to *Drosophila* circadian behavior. *Current Biology*, *20*(7), 600–605. https://doi.org/10.1016/j.cub.2010.02.044

Zhao, Z., Zhao, X., He, T., Wu, X., Lv, P., Zhu, A. J., & Du, J. (2021). Epigenetic regulator Stuxnet modulates octopamine effect on sleep through a Stuxnet-Polycomb-Octβ2R cascade. *EMBO Reports*, *22*(2), e47910. https://doi.org/10.15252/embr.201947910