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Simonetta Andreazza

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Analysis of new genes controlling *Drosophila melanogaster*
rest-activity rhythms

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Abbreviations

- AR: arrhythmic
- bHLH: basic Helix-Loop-Helix
- CaMKII: Calcium/calmodulin-dependent protein kinase II
- cDNA : complementary DNA
- CG#: *Drosophila* computed gene #
- ChIP: Chromatin IP
- *Cka/Cka*: Connector of kinase to AP-1 *gene/protein*
- CK2: Casein Kinase 2
- *Clk/CLK*: Clock (circadian locomotor output cycles kaput) *gene/protein*
- CREB: cAMP response element-binding protein
- *cry/CRY*: Cryptochrome *gene/protein*
- CT: Circadian Times
- *Cul3/Cul3*: Cullin3 *gene/protein*
- *cyc/CYC*: CYCLE *gene/protein*
- *cwo/Cwo*: Clockwork orange *gene/protein*
- *dcr2/Dcr2*: Dicer2 *gene/protein*
- *dbt/DBT*: Doubletime *gene/protein*
- DD: Dark-Dark
- DNA: Deoxyribonucleic acid
- DN: Dorsal Neurons
- dsRNA: double stranded RNA
- *strip/strip*: *Drosophila* Strip *gene/protein*
- EO: Evening Oscillator
- *frq/FRQ*: Frequency *gene/protein*
- GFP: Green Fluorescent Protein
- *gckIII/GckII*: Germinal center kinase III *gene/protein*
- H-B eyelet: Hofbauer-Bruchner eyelet
- *Hpo/Hpo*: Hippo *gene/protein*
- HS: Heat-shock (promoter)
- IP: immunoprecipitation
- *jet/JET*: JETLAG *gene/protein*

- *kay/Kay*: Kayak *gene/protein*
- LD: Light-Dark (cycles)
- LL: Light-Light
- l-LN_vs: large ventral LNs
- LN_ds: Dorsal LNs
- LNs: Lateral Neurons
- LPN: Lateral Posterior Neurons
- MAPK: Mitogen Activated Protein Kinase
- *msn/Msn*: Misshapen *gene/protein*
- MO: Morning Oscillator
- mRNA: messenger RNA
- *mts/Mts*: Microtubule star *gene/protein*
- *nmo/Nmo*: Nemo *gene/protein*
- NES: Nuclear Exporting Signal
- NLS: Nuclear Localization Signal
- OA: Okadaic Acid
- ORF: Open Reading Frame
- PAS: PER-ARNT-SIM (Period-Aryl hydrocarbon receptor nuclear translocator-Single minded)
- PCR: Polymerase Chain Reaction
- PDF: Pigment Dispersing Factor
- *Pdp1/Pdp1*: PAR-domain protein 1 *gene/protein*
- *per/PER*: Period *gene / protein*
- PP1: Protein Phosphatase 1
- PP2A: Protein Phosphatase 2A
- PTPase: Phospho-tyrosine phosphatase
- R: rhythmic
- Rh1-8: Rhodopsin 1-8
- RHT: Retinohypothalamic tract
- RNA: Ribonucleic Acid
- RNAi: RNA interference
- RT-PCR: Real-Time PCR
- s.d.: standard deviation

- s.e.m.: standard error of the mean
- SCN: Suprachiasmatic Nuclei
- *sgg*/SGG: Shaggy *gene*/protein
- *slmb*/SLMB: Supernumerary Limbs *gene*/protein
- s-LN_vs: small ventral LNs
- Strip: Striatin-interacting protein
- STRIPAK: Striatin-interacting phosphatase and kinase complex
- Strn: Striatin proteins
- *tim*/TIM: Timeless *gene*/protein
- *tw*s/Tws: Twins *gene*/protein
- USPs: Ubiquitin-specific processing proteases
- UTR: Untranslated Region
- *vri*/Vri: Vrille *gene*/protein
- WCC: White-Collar complex
- *wdb*/Wdb: Widerborst *gene*/protein
- ZT: *Zeitgeber* Time

Figure List

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INTRODUCTION

I. GENERAL ASPECTS OF BIOLOGICAL CLOCKS

1. Evolutionary advantages of having a clock

All living organisms are submitted to 24h day-night cycles that are imposed by the rotation of the Earth around its axis. They are also submitted to seasons as a consequence of the revolution of the Earth around the sun. These movements cause daily and seasonal fluctuations in light, temperature and humidity. It is thought that biological rhythms have evolved in response to those periodical geophysical changes and were fine-tuned under selective pressure. Indeed, great evolutionary advantages can be obtained if an organism has the possibility not to react but to *anticipate* those periodical changes for better fitting physiological and behavioral activities to the most favorable time.

Essentially all organisms studied so far, from cyanobacteria to human beings, show circadian rhythmicity. Pittendrigh's "escape-from-light" hypothesis proposed that circadian rhythms first originated in cells to protect the replication of DNA from ultraviolet radiation during daytime (Pittendrigh, 1993). However, some metabolic processes, like photosynthesis, require light. *Gonyaulax polyedra* is a unicellular alga that needs photosynthesis during the day and rises to the water surface shortly before sunrise. It migrates to great depth before sunset to take advantage of the higher nutrient concentrations and escape from short wavelength light. The internal clock lets incompatible metabolic processes to occur separately but also other essential physiological events to occur simultaneously. Producing an enzyme only at the time of need, for example, strongly reduces energetic costs.

Biological clocks are important at different levels of biological organization, from single cells to ecosystems. The clocks synchronize the daily activity of an organism to coincide with its ecological role, to be active when food is available and mating partners are active. Small nocturnal rodents, like mice, are active during nights and should return to their nest before the day starts and predators wake. Those behaviors require the synchronization of physiological processes to external signals. Abnormalities in the circadian clockwork are thus likely to harm the normal functioning of an organism in multiple ways (Merrow et al., 2005).

2. Historical background

The earliest known account of a circadian process dates as far as the 4th century BC, when Androstheneas, admiral of Alexander the Great, noted that the tamarind tree (*Tamarindus indicus*) opens its leaves during the day and closes them at night. For more than two millennia it was thought that such 24h cycles were directly governed by light. The first known experiment focusing on the nature of those biological rhythms was carried out in 1729 by the French astronomer De Mairan. The heliotrope “sensitive plant” *Mimosa pudica* folds its composite leaves every evening and unfolds them every morning. De Mairan noticed that the 24-hours pattern of the leaf movement continued even when the plants were kept in constant darkness. Thus, those daily leaf movements were not a direct effect of light exposure. Later on, similar daily opening and closing movements in leaves or flowers were observed. In the 18th century, the taxonomist Linnaeus even created a “floral clock”, in which every hour corresponds to a plant opening their flowers (Fig. 1).

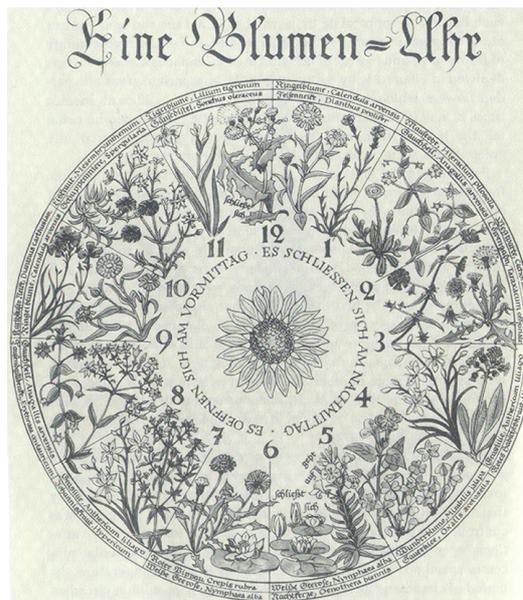


Figure 1 Representation of the Horologium Florae (Floral Clock) proposed by Linnaeus in 1751.

Each hour corresponds to the time of petal opening or closing for various species of flowers.

Evidence for the existence of an autonomous, internal “time device” came one-century later, in 1832, thanks to the Swiss botanist Augustin De Candolle. He observed that the plant *Mimosa pudica* had a period of leaf opening and closing of approximately 22-23 hours in constant light (*free-running* conditions), significantly less than the approximate 24 hour period of the Earth’s rotation period. This indicated that no environmental cues could have

entrained the rhythm, thus supporting an endogenous clock. In the early 1900's Erwin Bunning repeated Candolle's findings and came to similar conclusions: these rhythms were inherent to organisms and genetically determined. In fact, they persist generation after generation even when organisms (i.e. the bean plant *Phaseolus* or the fruit fly *Drosophila melanogaster* - (Pittendrigh, 1954)) were kept under constant dark conditions.

During the 20th century, circadian rhythms were described in many species (Fig. 2): rodents (1922, by C. Richter), insects (K. von Firsch and I. Beling), birds (1952, by G. Kramer) and humans (Aschoff and Wever, 1962). In 1953 Franz Halberg coined the term “circadian” combining the Latin words *circa* – meaning “around”, and *diem* – “day”, to describe rhythms with a period of about a day.

However, researchers in the 20th century were reluctant to accept the endogenous nature of the 24h rhythms. If not light, an unknown factor (“factor X”), like temperature, humidity or ionization of the air, must act instead of light. The conclusive evidence for the innate property of circadian rhythms came from transplantation studies. Transplanting a circadianly entrained tissue into a non-entrained one can induce circadian rhythmicity. Noticeably, other properties of circadian rhythmicity, such as phase and period, were transferred with the tissue transplantation (1972, independent experiments from R. Moore and I. Zucker).

Colin Pittendrigh conducted further in-depth studies on the characteristics of endogenous clocks using the eclosion (the emergence of the adult fly from its pupal case) rhythms of the fruit fly *Drosophila pseudobscura*. He demonstrated that rhythms in eclosion rates, which persisted in the absence of external light sources, can be reset or initiated (if started from a-periodic cultures of flies) by a single light pulse (synchronization), and were independent from temperature when changes occurred in a range of about 10°C (Pittendrigh, 1954). Only in the early 70s the first clock mutants were identified: the *period* locus in *Drosophila melanogaster* (Konopka and Benzer, 1971) and *frequency* in the fungus *Neurospora crassa* (Feldman and Hoyle, 1973). These experiments trigger numerous subsequent studies on the molecular basis of circadian rhythms. The “transcriptional/translational feedback loop model” was first described in *Drosophila* (Hardin et al., 1990) and orthologs of its main components were found in other organisms, in particular mammals.

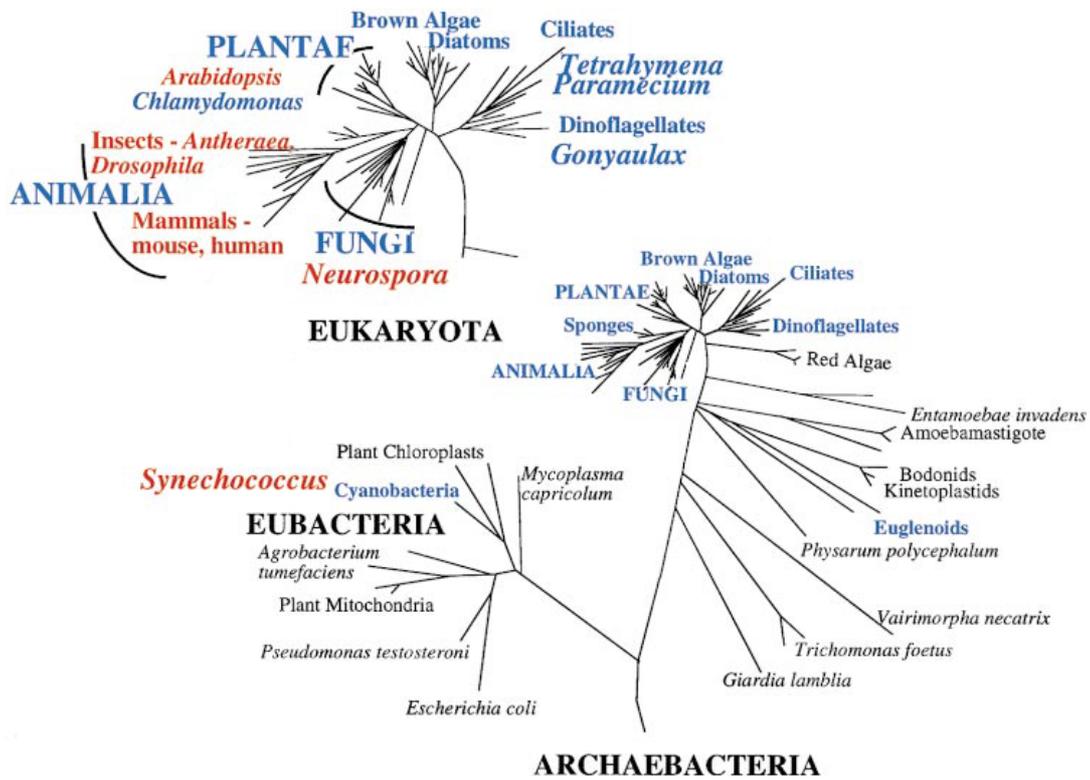


Figure 2 Circadian systems in the tree of life

The diagram shows the range of organisms in which circadian systems have been investigated. In blue are phylogenetic groups in which circadian rhythms have been described (at least at the physiological level); in red are the systems in which the clock mechanism has been studied in depth, at a genetic and molecular level. The scaled line lengths correspond to evolutionary distance between phylogenetic groups (Dunlap, 1999).

3. Characteristics of a biological clock

Clocks that are found within a living organism are called “biological clocks”.

a. Rhythms: period, phase and amplitude

As for any rhythmic process, biological oscillations are characterized by period, phase and amplitude. The period indicates the duration after which a defined point of the oscillation re-occurs. The phase defines the instantaneous state of the oscillation, compared to a reference point; hence, it describes the relationship between a given reference point in the rhythm and the entraining cycle. The amplitude indicates the difference between the maximum (or minimum) and the mean value of the rhythmic parameter, and defines the robustness of the rhythm.

b. Circadian clock properties

Circadian clocks, the most exhaustively studied natural timekeeping devices, turn with a period of about (*circa*) a day (*diem*). Circadian clock are characterized by the following properties:

1. They are *self-sustained*. They keep ticking even in the absence of external cues, meaning without any “time-giver” event – like temperature or light, with a period approximate to 24h. Kept under constant conditions, the rhythm starts to *free-run*, which means that it is not synchronized anymore by the environment.
2. They *synchronize to and can be entrained by* environmental cues, called *zeitgebers* (from German, “time-givers”). The rhythm can be reset by exposure to external stimuli (such as light): this is essential to adjust to the local time. A person who travels across time zones will usually experience jetlag effects before its internal clock entrains to and come *in sync* to the local time.
3. They are *temperature-compensated*. Organisms live at a broad range of temperatures, and temperature strongly affects the kinetic of molecular processes. However, compensated clocks have approximately the same period length within the range of physiological temperature.

c. The “clock model”: input – pacemaker – output.

Clocks are usually defined as “mechanical or electrical device for measuring time, indicating hours, minutes, and sometimes seconds by hands on a round dial or by displayed figures” (<http://oxforddictionaries.com>). From a biological point of view, a clock represents an endogenous, genetic timekeeping mechanism, able to perceive informations from the environment, integrate it, and then coordinate multiple physiological and metabolic outputs. We consider a system that oscillates independently as a “master clock” or “pacemaker”; conversely, if the oscillations depend on the oscillations of a superior system, that oscillator is called a “slave clock”.

Circadian clocks are usually defined by three essential elements (Fig. 3a):

- input pathways (orange), that sense synchronizing environmental cues and transmit them to the circadian oscillator;
- a circadian oscillator (white), that keeps circadian time and activates output pathways;

- output pathways (violet), that transfer temporal informations from the oscillator to orchestrate diverse physiological, metabolic or behavioral responses.

Input environmental signals, mostly light and temperature – but also food intake and other social, physical or chemical factors, are received via receptors and then sent to the central pacemaker that generates oscillations. The output pathway(s) translates the oscillation into overt rhythms such as genome transcription in cyanobacteria or sleep-wake cycles in animals. Nevertheless, this linear progression from input to output is an over-simplification, at least in higher organisms (Fig. 3b). In most of commonly studied organisms, rhythmic output activities may feedback and function themselves as positive - or negative input cues for the functioning of the central pacemaker. That’s the case, for example, of melatonin secretion from the pineal gland, which may act, upon specific receptor, back to the clock. Moreover, multiple interlocked oscillators co-exist at the master level and integrate input cues from the outside (Gardner et al., 2006).

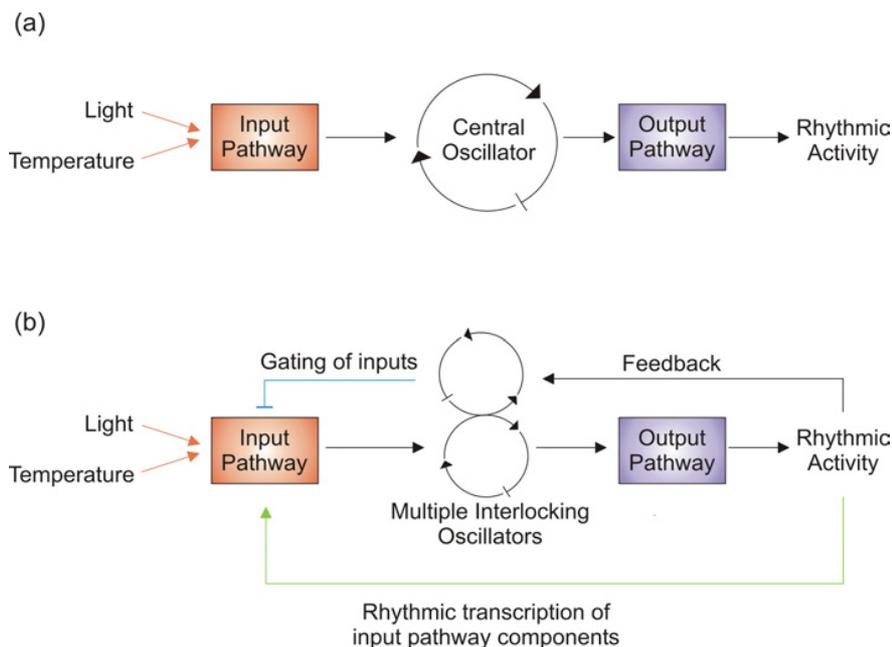


Figure 3 The “clock model”.

(a) A model describing the structural organization of the circadian timing system into an input pathway, a central oscillator, and an output pathway. (b) An elaborated description of the clock: multiple core oscillators, gated input pathways and outputs that feed back into the central oscillator (Gardner et al., 2006).

II. INPUTS AND OUTPUTS

While period is ~24h in the absence of environmental cues, circadian oscillations achieve a precise 24h period by adjusting to recurring external signals such as solar light. The mechanism by which the clock sets its time according to the external cues is called *entrainment*, and the environmental signal that can phase-set the circadian clock is called *zeitgeber*.

Importantly, external cues can both entrain and mask the rhythm. Entrainment affects the phase of the endogenous oscillator, which controls overt behaviors. Masking arises from a direct effect of the external cue over a behavioral output and does not affect the phase of the oscillator.

1. Light, temperature and other zeitgeber

a. Light perception

Light appears to be the strongest entraining signal for circadian clocks. Almost all organisms have evolved receptors to capture light information from the environment.

In mammals, the light stimulus is only sensed through the eyes and their bilateral removal completely abolishes photo-entrainment. Light information is mainly perceived (but rod and cone photoreceptors are also involved) *via* intrinsically photosensitive retinal ganglion cells (ipRGCs) in the inner retina, which express the photopigment melanopsin. These cells project directly to the suprachiasmatic nuclei (SCN), the brain “master clock” (see § III.1.a), through the retinohypothalamic tract (RHT) (Albrecht, 2012).

In contrast to mammals, *Drosophila* perceives light stimuli through two different pathways: rhodopsin-expressing photoreceptors (compound eyes, Hofbauer-Buchner eyelet, ocelli) and CRYPTOCHROME, a deep-brain blue-light photoreceptor protein (Fig. 4). The specific contribution of each of those light input pathways to clock entrainment is only partially resolved.

The fruit fly possesses six types of rhodopsins. Each rhodopsin responds to light with a different peak of absorption, together covering a wide range of wavelengths (maximal sensitivities go from around 300 to 550 nm). The visual system is sufficient but not necessary for flies to entrain to light-dark cycles (Ashmore and Sehgal, 2003). Cryptochromes (CRYs)

are blue-light sensitive flavoproteins involved in the synchronization of plant and insect clocks by light. *Drosophila cry* is expressed in (a large subset of) the brain neurons, including “clock” neurons - that coordinates *Drosophila* rhythmic locomotor behaviors, and in peripheral tissues, such as the eyes, gut and antennae. Light induces a conformational change in the CRY protein that directly resets the molecular clockwork through binding to one of its components, TIMELESS (TIM) (see § II.2). In contrast to visual light input pathways, CRY signaling is able to induce phase resetting even after short pulses of light (Ivanchenko et al., 2001).

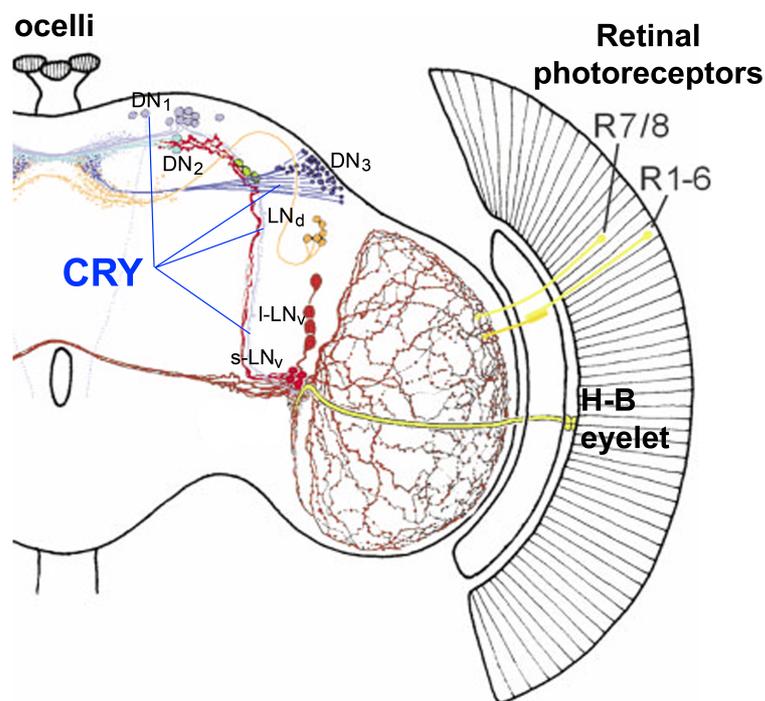


Figure 4 Light input pathways to the *Drosophila* brain.

The fruit fly perceives light through rhodopsins activation in the visual system (compound eyes), the Hofbauer-Buchner (H-B) eyelet and the ocelli. The photoreceptors of the compound eyes include five types of rhodopsins. Rhodopsin 1 (Rh1) is expressed in R1-6 photoreceptors, Rh3 or Rh4 in R7 photoreceptors, and Rh5 or Rh6 in R8 photoreceptors. Rh6 is also expressed in the H-B eyelet, a sub-retinal structure of the fly optic lobe that sends projections to the central brain. Rh2 is specifically expressed in the photoreceptors of the three ocelli that are located on the top of the head. In addition, the blue-light photosensitive CRYPTOCHROME (CRY) protein is expressed by the majority of the brain clock neurons (LN_vs, 3/6 LN_ds, DN₁, DN₃) and allows direct light perception. Adapted from Helfrich-Forster, 2003.

b. Temperature sensing

In addition to light, temperature functions as a key entraining agent for many ectothermic organisms, like insects. Flies mutant for either the visual system or *cryptochrome* still entrain to temperature cycles. Cells are expected to directly sense temperature variations, hence, synchronization by temperature is presumably a tissue-autonomous process. However, clock neurons synchronization under temperature cycles requires some “entraining signals” coming from the periphery (Glaser and Stanewsky, 2005). Hence, in contrast to peripheral tissues, temperature entrainment in the central brain clock neurons seems to be non cell-autonomous. Chordotonal (ch) organs, which are mechano-sensory organs located just below the cuticle, and the gene *nocte* were proposed to play a role in temperature entrainment (Sehadova et al., 2009). Importantly, temperature, more than light, has recently been shown to influence the fruit fly activity in outdoor natural conditions (Vanin et al., 2012).

In mammals, as in mainly endothermic vertebrates, environmental temperature variations exert very little effects, although internal temperature cycles play a major role in the synchronization of peripheral clocks.

Remarkably, a key property of circadian rhythms is that their period is temperature compensated – meaning that the period of the rhythm remains constant whether temperature increase or decrease within physiological ranges (Pittendrigh, 1993). However, daily variation in temperature can be used to phase-set the oscillation.

c. Other zeitgebers

In addition to light and temperature, the body clocks use several other external stimuli to synchronize the internal to external time. According to the different organisms’ niche, these signals can include tides, moonlight, social contacts and food availability and intake. Experiments have shown that social interactions among flies can transmit informations about time, and this process involves olfactory cues (Levine et al., 2002b). Feeding cycles are an important *zeitgeber* for some peripheral clocks (§ III.1.a) in mammals (Schibler, 2005).

2. Light-mediated synchronization and phase resetting

Among all *zeitgebers*, light appears to be the strongest *zeitgeber* for the resetting the brain clock. The perception of the entraining signal (light) at a defined point in the oscillation produces a variation in an oscillating parameter that feeds-back (phase advance) or feeds-forward (phase delay) the oscillation program to a different point.

In mammals, phase resetting in the SCN clock (see § III.1.a) is achieved through the light-mediated transcription of relevant clock genes. In brief, light perception stimulates the release of glutamate (among other neurotransmitters) from the axons of the retinohypotalamic tract (RHT) to the neurons of the suprachiasmatic nuclei (SCN). The calcium influx that follows triggers the activation of diverse signal transduction cascades, including calmodulin kinase II (CaMKII) and mitogen-activated protein kinase (MAPK), all converging in the phosphorylation of cAMP-response element-binding protein (CREB). Phospho-CREB in turn activates transcription of the clock genes *per1* and *per2* by binding to CRE element in their promoter regions (Golombek and Rosenstein, 2010).

While mammalian phase resetting is primarily governed by a transcriptional regulation, entrainment in the *Drosophila* clock through CRY is first based on posttranslational responses. Upon light exposure (even of short light pulses), the TIMELESS (TIM) protein - one of the major components of the fruit fly molecular clock machinery (see § IV.1), is rapidly (within 30 min) degraded via the proteasome system (Zeng et al., 1996). Nevertheless, TIM is not itself a photoreceptor. Light affects TIM through the activation of the blue-light-sensitive CRY protein (Fig. 5). Light induces a conformational change in the CRY protein that allows it to bind TIM. TIM is then phosphorylated by a yet uncharacterized Tyr-kinase (pharmacological studies by Naidoo et al., 1999) and interacts with the JETLAG (JET) ubiquitin ligase, which irreversibly targets TIM for degradation by the proteasome (Koh et al., 2006); a functional COP9 signalosome appears required (Knowles et al., 2009). The CRY protein also binds JET in a light-dependent manner, and this interaction is followed by CRY proteolysis (Peschel et al., 2009); however, JET implication in CRY degradation has not been confirmed further. Instead, knockdown of BRWD3/Ramshackle, known to confer substrate specificity to a Cul4-based ubiquitin ligase complex, strongly curbs light-mediated CRY degradation (Ozturk et al., 2013). In addition to a key role for the light entrainment, the CRY protein appears to function as a repressor within the core feedback loop underlying circadian rhythms (see § III.1.b) in several *Drosophila* peripheral tissues.

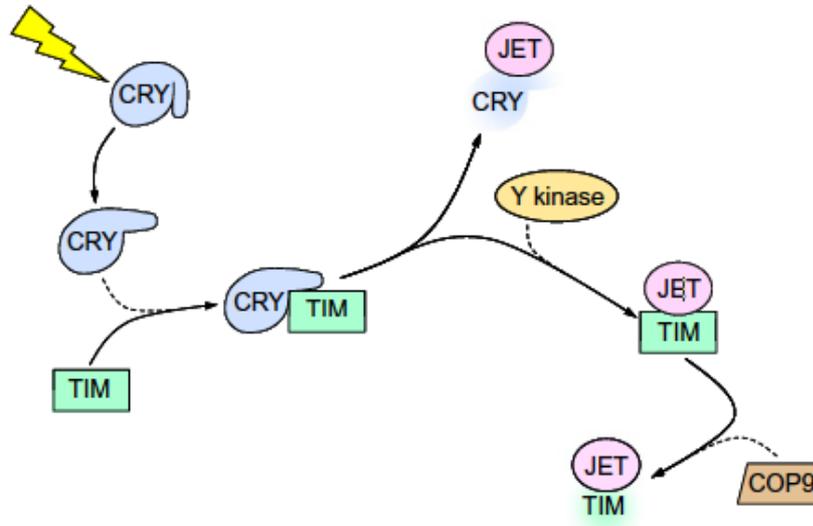


Figure 5 Light-induced phase resetting in *Drosophila*.

Light triggers a conformational change in the CRY photoreceptor that allows CRY binding to TIM. TIM is then phosphorylated by a yet uncharacterized Tyr (Y)-kinase. TIM and CRY are recognized by the JET ubiquitin ligase that targets TIM for degradation through the proteasome. The COP9 signalosome is also required (Hardin, 2011).

3. How to study circadian clocks: measurable outputs

To study circadian rhythmicity in organisms we have to assess measurable outputs. In addition to many oscillating biochemical parameters, including body temperature, hormones release and metabolic activities, rhythms in gene expression and overt behaviors are evaluated. Luciferase fusion transgenes are used to monitor rhythms of gene expression in the unicellular cyanobacterium *S. elongatus*. The fungus *Neurospora crassa* produces asexual spores under the control of biological clocks and this process of conidiation can be measured using specialized growth chambers called “race tubes”. For rodents, locomotor activity rhythms are commonly used to evaluate alteration of the circadian setup by measuring daily wheel-running activity.

Circadian outputs in the fruit fly: locomotor activity rhythms

The circadian oscillator controls several aspects of the *Drosophila* life, including the timing of courtship behavior and egg laying, or physiology (olfactory responses). Pupal

eclosion (emergence of adult flies) is also under circadian influence, and led to the discovery of the first clock mutants (Konopka and Benzer, 1971). Locomotor activity rhythms are currently the easiest and most used readout of the *Drosophila* circadian clock (Dubruille and Emery, 2008).

Locomotor activity of single flies is recorded by the use of activity monitors housed in light- and temperature-controlled incubators (see ANNEXES § I). Bouts of activity are depicted as vertical black bars during a 48h time space in double-plotted *actograms*, which provide informations about the period and phase of the internal clock. *Eductions* show the average daily activity of flies in a 30-min unit and describe activity with a better resolution, in particular to estimate anticipatory behaviors (Fig.6). In light-dark (LD) cycles (12h of light followed by 12h of darkness at constant room temperature conditions), *Drosophila* flies show a bimodal activity profile and are “crepuscular”. They show a bout of activity around the lights-OFF to lights-ON transition (referred as the *morning peak*). After a “siesta” in the mid-afternoon, a second burst of activity occurs around lights-ON to lights-OFF transition (*evening peak*) (Fig. 6). Once placed in constant dark conditions (DD), flies continue to show a 24h rhythmic pattern of activity, but morning and evening peaks progressively fuse to a unique and broader bout of activity at the end of the subjective day (i.e. the time when light would normally be present in LD). In constant light conditions (LL) wild-type flies are completely arrhythmic (AR).

Remarkably, flies easily adapt to a shift in the time schedule of light, and in three days a wild type fly is able to synchronize to a 12h-phase shift (anti-phase). Disruption or alteration of the clock inherent periodicity can be easily visualized in an actogram (Fig. 7). Upon establishment of constant conditions (constant darkness together with constant temperature), animals loose any reference to the external time schedule and behave solely according to the time indicated by their internal clock. Flies without a functional clock (like *per*⁰ mutants) show randomly distributed activity or arrhythmicity. Flies with a period shorter than 24h (i.e. *per*^{short} mutants) start to be active sooner every day and activity bars in the actogram shift towards the left. Conversely, long-period (>24h) animals (i.e. *per*^{long} mutants) show activity that moves day by day rightward.

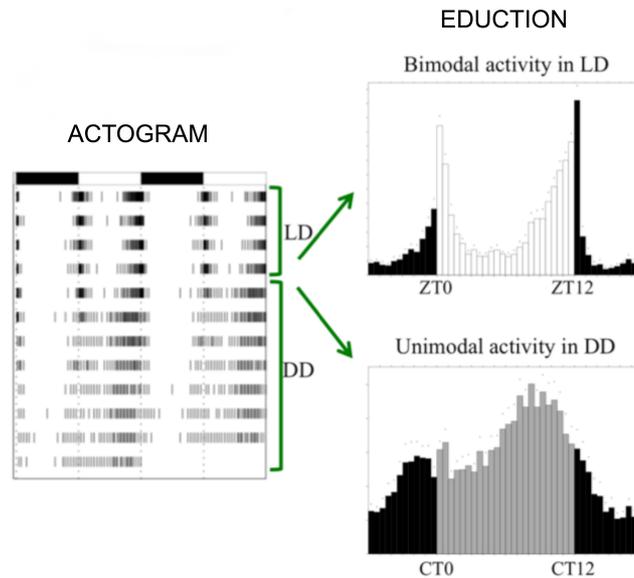


Figure 6 *Drosophila melanogaster* displays a bimodal activity in light-dark (LD) conditions (first 5 days) and a unimodal activity in DD conditions (last 7 days) at 25°C. Graphs show the average activity of a group of male flies. The actogram (on the left) represents the average activity pattern during subsequent days (two subsequent days in a row, and subsequent days in a column). Day and night (real or subjective) are shown on the top by white and black boxes, respectively. The density of black bars in the actogram is proportional to the activity of the fly. Educations (on the right): each box in the histogram represents the average locomotor activity of the flies during 30 min. ZT0 and ZT12 are the times of lights-on and lights-off, respectively. CT0 and CT12 mean the times of the lights-on and -off during the entrainment before the constant darkness conditions.

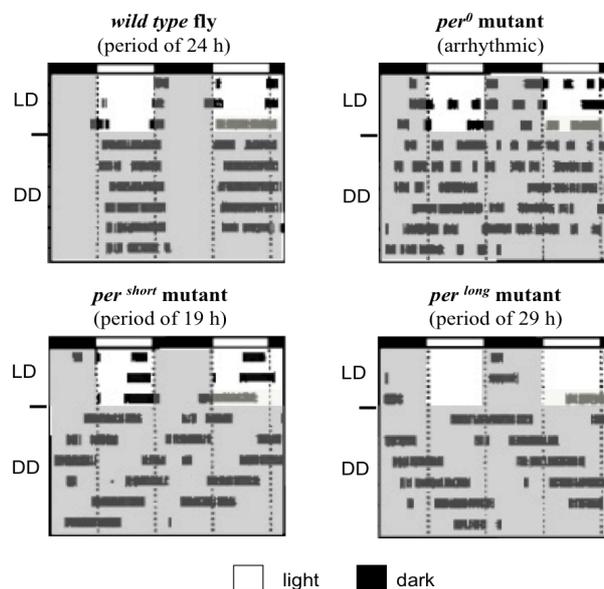


Figure 7 Actograms of arrhythmic flies (per^0), short-period flies (per^{short}) and long-period flies (per^{long}). *Period* is the first clock gene that has been isolated. By chemical mutagenesis, S. Benzer and R. Konopka isolated three different alleles at a single locus (*period*) that drastically affected the locomotor activity rhythm of flies, inducing the appearance of arrhythmic behaviors (per^0), or rhythmic behaviors but with a period shorter (per^{short}) or longer (per^{long}) than 24h (Konopka and Benzer, 1971).

III. ANATOMICAL ORGANIZATION OF THE CLOCK

It is believed that almost every cell of an organism's body contains a circadian clock that functions in an autonomous manner. However, to coordinate proper biochemical and physiological functions, each cell of a tissue must work cooperatively and simultaneously, hence cells must be synchronized within the tissue. Moreover, a precise phase-relationship within different tissues and organs is needed to organize more complex and timely regulated responses at the system level. In addition, diverse, separated oscillators likely coordinate day and night overt behaviors (Pittendrigh, 1993).

1. Central and peripheral clocks

Not every cell is able to perceive and/or to respond to a certain given stimulus. Time informations and phase coordination is thus held by the functions of a "central" time-giver system that integrate multiple input signals and transmit phase informations to downstream tissue-specific clocks. We can thus discern between a brain *master clock*, which can entrain autonomously and generate self-sustainable robust rhythms, and "peripheral" *slave clocks*, that receive time informations from the central clock and refer to it for phase resetting and long-term rhythm sustaining.

a. In mammals

By the use of anatomical (direct target of the retinal fiber) and surgical (ablation and transplantation) approaches the mammalian central pacemaker was isolated in the nuclei located just above the optic chiasm within the hypothalamus region, hence named the suprachiasmatic nuclei (SCN). The SCN integrates light input informations and a number of different input signals converging to the SCN from separated brain and body regions (Fig. 8). The circadian oscillations generated in the SCN are converted into rhythmical release of hormonal and neuronal signals that are sent to peripheral tissues outside the SCN (including liver, heart, lung and other brain regions). These informations then directly or indirectly phase-sets peripheral clocks.

Unlike the central oscillator (SCN), the mammalian peripheral oscillators are not directly entrained by light. In addition to SCN-derived time signals, peripheral oscillators use alternative stimuli, like the feeding-time for the liver clock, as greater *zeitgeber*.

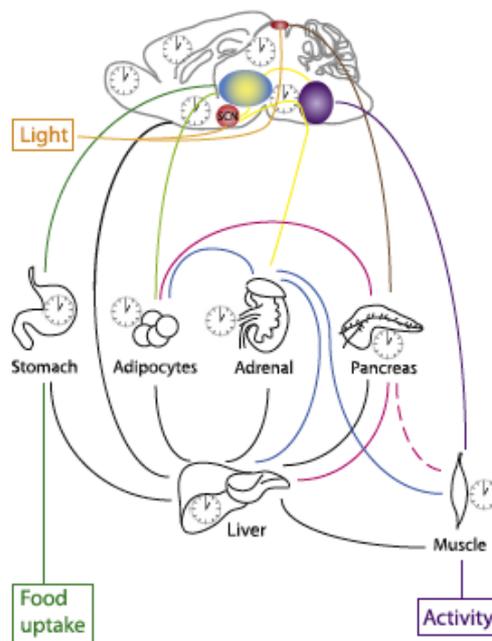


Figure 8 The mammalian circadian system.

The mammalian master clock is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (red), which directly integrates light informations from the eyes and other non-photoc entraining signals (directly or indirectly) from peripheral tissues. Oscillations in the master clock synchronize the clocks of peripheral tissues. Peripheral clocks also directly perceive synchronizing (non-photoc) cues from the environment (Albrecht, 2012).

b. In Drosophila

In contrast to mammals and thanks to the transparency of the fly cuticle, almost every single cell in *Drosophila* can detect light directly or receive light informations from photoreceptor cells. Indeed, a *per* promoter-driven (*period*, *per*, is a major clock gene) *luciferase* or *green fluorescent protein* reporter gene (*per-luc* or *per-gfp*) shows bioluminescence oscillations in the whole fly as well as in isolated heads, thoraxes or abdomens, and those oscillations clearly show light-mediated phase adjustment (Fig. 9) (Plautz et al., 1997). Therefore, we cannot define a “master clock” in *Drosophila* that entrains peripheral oscillators but each cell functions as a self-sustained, main oscillator.

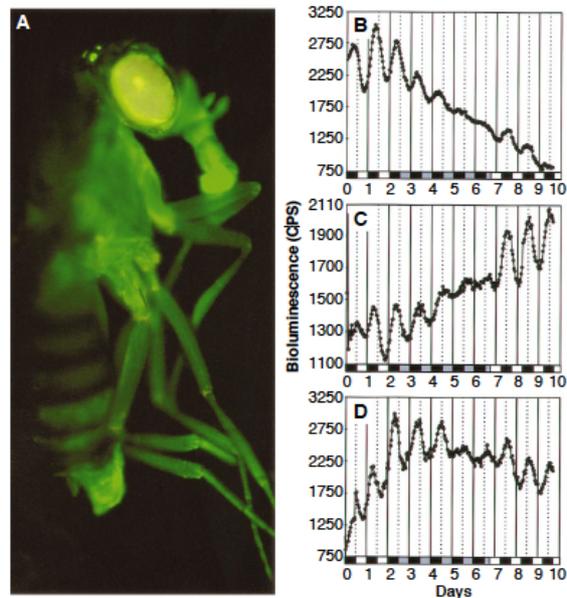


Figure 9 Bioluminescence rhythms in *Drosophila* peripheral oscillators.

Oscillation of a *luc* reporter under control of the *per* gene promoter sequences can be seen throughout the whole fly (A). Bioluminescence oscillations persist in separated heads (B), thoraxes (C) and abdomens (D) individually cultured in LD (light and dark boxes) and DD (grey boxes: subjective day; black boxes: subjective night) conditions (Plautz et al., 1997)

Several pieces of evidence have suggested that CRY possesses some photoreceptor-independent activities in (at least some) peripheral tissues, where it is required for oscillator function *per se*. *cry^b* (*cry^{baby}*) flies, bearing a severe hypomorphic mutation at the *cry* locus, can be entrained by temperature cycles but do not respond to light-dark cycles (Stanewsky et al., 1998). Consistent with this, bioluminescence rhythms in *cry^b* flies bearing a *per-luc* reporter still entrain to temperature cycles in the eye or wing tissues. Conversely, daily cycles in electroantennogram (EAG) responses to odors in those flies were severely disrupted not only under LD cycles but even when flies were exposed to temperature cycles. Bioluminescence analyses revealed that flies had lost oscillation of the *per-luc* reporter expression in antennae: loss of CRY function(s) had impacted the core feedback loop work (Krishnan et al., 2001). Similar results were reported for clock gene expression in the foreleg and Malpighian tubules (the fly kidney equivalent) (Ivanchenko et al., 2001; Levine et al., 2002a). Similarly, expression of many clock genes was constantly near peak levels in head extracts (where the large majority of circadian mRNA expression is from the peripheral oscillators of the eye) of *cry^b* flies (Collins et al., 2006). This is consistent with a role for CRY

as a transcriptional repressor in the eyes, similar to mammalian CRY1 and CRY2 functions (§ V.2).

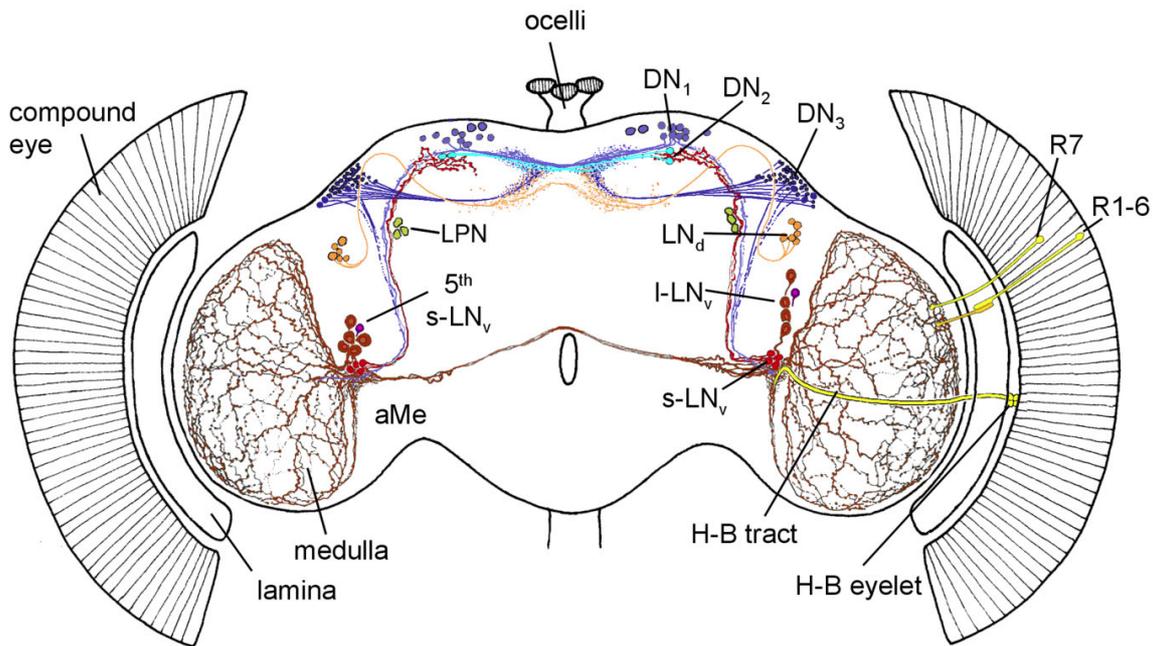
Although *Drosophila* tissues display autonomous oscillator functions, pacemaker cells in the fruit fly brain directly control the phase and periodicity of overt locomotor activity rhythms, and as such may be considered as a “central oscillator”.

The “central clock” of *Drosophila* adult flies relies on about 70-80 neurons in each brain hemisphere, organized in different groups and named accordingly to their location, morphology and/or properties (Fig. 10A).

The clock neurons are bilaterally clustered into two main groups: the dorsal neurons (DNs) that lay in the dorsal protocerebrum, and the lateral neurons (LNs) located between the optic lobe and the central brain. Four groups of LNs are located in the anterior brain and one in the posterior brain. The LNs of the anterior brain are subdivided into the dorsal LNs (LN_{ds}, six cells), the large ventral LNs (l-LN_{vs}, four or five large cells) and the small ventral LNs (four s-LN_{vs} and the 5th s-LN_v). The group of LNs located in the posterior brain is called LPN (lateral posterior neurons, four cells). The DN_s are the most abundant in number and consist of three main groups – the DN_{1s} (fourteen to sixteen cells), DN_{2s} (two cells) and DN_{3s} (about forty cells each hemisphere). In the *Drosophila* larval brain only the five s-LN_{vs}, the two DN_{2s} and two DN_{1s} are found (Fig. 10B).

Neurons within each group show common clock properties but differentiate each other in term of input responsiveness (light and or temperature sensitivity), oscillating properties, output projections and neurotransmitter(s) release. The s-LN_{vs}, LN_{ds} and DN_s neurons show strong (clock) protein cycles in light-dark conditions, and oscillations persist with the same phase relation for several days in constant darkness. On the contrary, l-LN_{vs} do not free-run (i.e. do not oscillate) in constant darkness, and their circadian function is questioned. Neurotransmitters mediate communication among those different groups of neurons. Glutamate, acetylcholine and several neuropeptides (PDF, NPF, ITP among others) appear to be involved. The l-LN_{vs} and the four s-LN_{vs}, but not the LN_{ds} and the 5th s-LN_v express the pigment-dispersing factor (PDF) neuropeptide, which is required for rhythmic free running locomotor activity (Nitabach and Taghert, 2008).

A



B

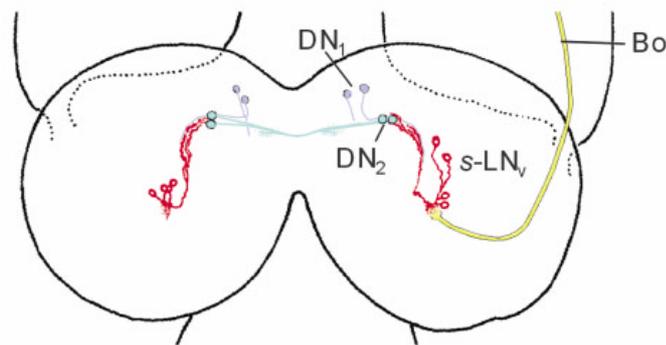


Figure 10 Organization of the “central clock” neuronal network in a *Drosophila* adult fly brain

(A) and a larval brain (B). (A) The adult fly clock network consists of 150 neurons bilaterally organized in groups according to their anatomical location. The lateral neurons (LNs) consist of the LN_d cells (orange), the PDF-positive I-LN_v and s-LN_v cells (red), and the PDF-negative 5th s-LN_v cell (violet). The dorsal neurons (DNs) comprise the DN₁, DN₂, and DN₃ cells (all in blue). Furthermore, three PER/TIM neurons are located in the posterior lateral brain (lateral posterior neurons; LPN, in green). The PDF-positive s-LN_v and I-LN_v cells send fibers into the accessory medulla (aMe) and medulla, where they likely receive light inputs from photoreceptor cells of the compound eyes and from the Hofbauer-Bucher (H-B) eyelet. Virtually all clock neurons with known projections send axons to the dorsal protocerebrum, where their terminals largely overlap with each other. (B) The larval clock network consists of only eighteen neurons: five s-LN_vs and two pairs of DN₂s (that project controlaterally) and two DN₁s. Four out of the five s-LN_vs express PDF and are connected to the Bolwig’s organ, the larval photoreceptive organ (Helfrich-Forster 2003).

2. Morning and evening oscillators

The fruit fly is a crepuscular animal: it is predominantly active at dawn and dusk. This bimodal distribution of locomotor activity, foreseeing the variation of one such strong *zeitgeber* (light), was found in many other organisms, mammals included. In 1976, Pittendrigh and Daan (Fig.11) noticed that the activity pattern of mice adjusted to the day length (photoperiod). They suggested the existence of two distinct circadian oscillators: one controlling the morning activity (morning oscillator, MO) and the other regulating the evening peak (evening oscillator, EO). MO and EO opposite respond to illumination: light shortens the period of the MO, while it lengthens the period of the EO (Pittendrigh and Daan, 1976).

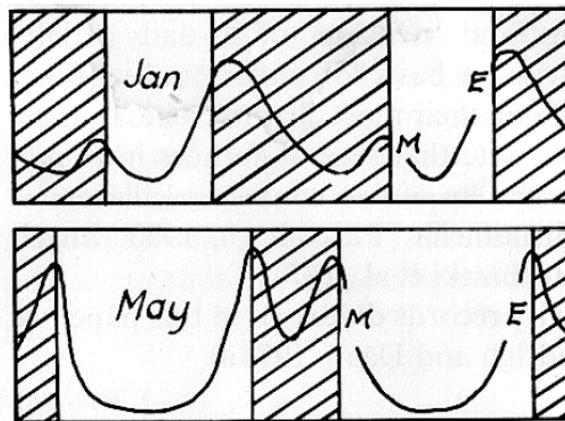


Figure 11 Bimodal distribution of locomotor activity of *Mus musculus* under short (top panel) or long (bottom panel) photoperiods – January and May. M: morning activity, E: evening activity (Pittendrigh and Daan, 1976)

In *Drosophila*, the four s-LN_v neurons control the morning anticipation program (MO) (Cusumano et al., 2009), while the 5th s-LN_v, LN_ds and some DN₁s house the EO that controls evening anticipatory activities. Both functional restoration studies (UAS/GAL4-mediated *per* expression in a *per*⁰ mutant background, Grima et al., 2004) and targeted ablation studies (Stoleru et al., 2004) revealed this discrete pattern of functional organization of clock neurons.

In addition, the PDF-positive s-LN_vs were shown to be necessary and sufficient to sustain rhythmicity in constant dark conditions (Grima et al., 2004; Nitabach and Taghert, 2008). Conversely, the EO is sufficient to drive rhythmic locomotor activity in constant light (Picot et al., 2007; Stoleru et al., 2007). Morning and evening oscillators are thus differently affected

by light. Data are consistent with light promoting evening activity and day rhythmic behaviors, while it inhibits morning anticipation and night rhythmicity (Picot et al., 2007).

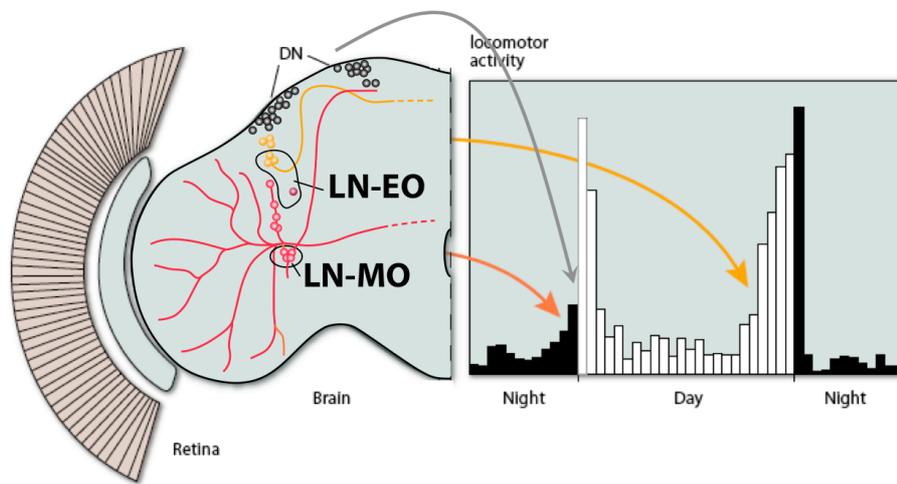


Figure 12 Neuronal bases of the morning (MO) and evening (EO) oscillators.

LN-MO: four PDF⁺ s-LN_vs (red); LN-EO: 5th s-LN_v (red) and three CRY⁺ LN_ds (yellow) (Cusumano et al., 2009).

Several behavioral and biochemical data support the existence of a crosstalk between neurons of the morning and the evening oscillators, with PDF (exclusively secreted by the LN_vs) influencing the entire clock network (Cusumano et al., 2009).

In this chapter the anatomical localization of central/peripheral clocks and properties of morning/evening oscillators have been discussed. Although they manifest differences in sensitivity to light and other time-cues, rhythm robustness and tissue-specific output pathways, the molecular bases underlying both central and peripheral circadian clocks are very similar and roughly shared among very different organisms. The next chapter (§ IV) will illustrate how circadian rhythmicity is maintained at the molecular level.

IV. CIRCADIAN OSCILLATORS: THE MOLECULAR MACHINERY

In eukaryotes the circadian oscillator is composed of interconnected transcriptional-translational feedback loops. These loops produce coordinated rhythms in “clock” genes transcription and “clock” proteins modifications, which finally sustain the daily progression of the clock machinery.

As a general rule, positive and negative regulators can be defined (see Fig. 16A). Positive elements (transcription factors) enhance the expression of gene(s) encoding one or more negative regulators. The synthesized repressor protein(s) enter the nucleus and, after some delay, inhibit the activity of the transcription factor complex, repressing their own synthesis. Finally, disruption of negative elements releases inhibition and allows initiation of a novel cycle (Tataroglu and Schafmeier, 2010).

1. The *Drosophila* molecular clock

In *Drosophila*, two interlocked feedback loop have been described (Yu and Hardin, 2006): the main *period/timeless* feedback loop, that controls the transcription of negative regulators; and the *Clk* feedback loop, that controls the expression of the *Clock* transcription factor – one of the two positive regulators. These loops organize oscillations in the transcription of circadianly controlled “clock” genes and contribute to the cycling of the related clock proteins (Hardin, 2011).

a. The per/tim feedback loop

During the late day, the positively acting basic helix-loop-helix (bHLH)/PAS (PER, ARNT, SIM) transcription factor partners CLOCK (CLK) and CYCLE (CYC) bind to E-box enhancer elements and activate transcription of the core clock genes *period* (*per*) and *timeless* (*tim*) - among other target genes (Fig. 13) Although *per* and *tim* mRNA levels accumulate during this phase, PERIOD (PER) and TIMELESS (TIM) proteins do not. The TIM protein is sensitive to light: light-activated CRYPTOCHROME (CRY) recognizes TIM and then JETLAG (JET), an ubiquitin-ligase enzyme, induces its degradation during the light-phase. In constant darkness conditions this process is carried out by a Cullin-3 (CUL3)-based ubiquitin

ligase (Grima et al., 2012). Newly synthesized PER in the cytoplasm is rapidly recruited by the DOUBLETIME (DBT) kinase, which phosphorylates PER and targets it for proteasomal degradation. Recent data (Lim et al., 2011) suggest that translational regulation may also participate to the delay in PER cytoplasmic accumulation. During this phase, PER is also recruited by the CK2 kinase (CK2) – which is believed to be a priming kinase for DBT activity, and PP2A phosphatase (Sathyanarayanan et al., 2004). TIM is required for the phosphorylation of PER by CK2 (Meissner et al., 2008) and interacts with PP1 phosphatase (Fang et al., 2007). Likely, coordinated activities of kinases and phosphatases maintain low levels of hypo-phosphorylated PER and TIM proteins at the beginning of the night.

As the level of TIM increases after sundown, TIM binds PER and somehow prevents PER degradation, despite PER is still bound and phosphorylated by DBT. Such stabilized PER/DBT/TIM complexes aggregate in the cytoplasm (Meyer et al., 2006), together with other partner enzymes (CK2, SGG, other still unknown kinases, PP1 and PP2A) that further modify PER and TIM proteins. These modifications account for the molecular weight shift of PER and TIM proteins on Western blots. Phosphorylation of TIM by SHAGGY (SGG) and PER by CK2 promotes PER/DBT and TIM entry into the nucleus. The cytoplasmic complex likely dissociates before this step as PER/DBT and TIM are thought to enter the nucleus separately, with PER/DBT nuclear appearance happening just before that of TIM (Meyer et al., 2006). In the nucleus, PER/DBT and TIM bind to CLK/CYC and repress transcription of *per*, *tim* and other target genes by removing CLK/CYC from chromatin (Yu et al., 2006; Menet et al., 2010). Transcriptional repression is achieved by the formation of a “repressor” complex that contains multiple partners. During this step, CLK is hyper-phosphorylated, a condition associated to its inability to initiate transcription.

Just before and mainly after sunrise, levels of TIM and PER undergo strong decrease due to proteasomal degradation. Light induces CRY-mediated TIM breakdown. PER undergoes DBT-directed hyper-phosphorylation that allows PER recognition by the ubiquitin ligase SLMB and proteasome-mediated proteolysis, even in the absence of light cues. In contrast to PER and TIM, the quantities of which fall during the day, the overall CLK quantities do not decrease thanks to the supply of newly synthesized hypo-phosphorylated CLK. CLK dephosphorylation by PP2A also contribute to stabilization of a transcriptionally active hypo-phosphorylated CLK form (Kim and Edery, 2006). Hypo-phosphorylated CLK heterodimerizes with CYC and binds to E-boxes to initiate another cycle of transcription.

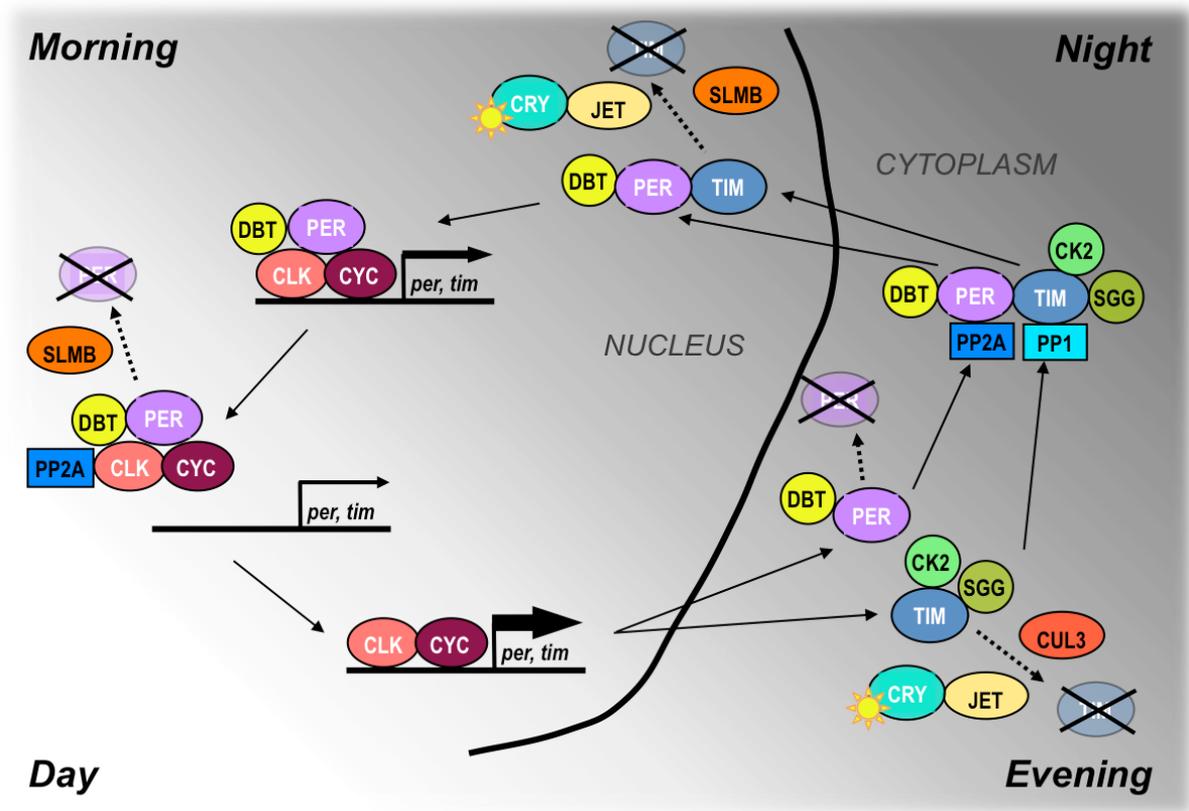


Figure 13 The *per/tim* feedback loop.

At the end of the day, the CLOCK (CLK)/CYCLE (CYC) heterodimer drives transcription of the *per* and *tim* genes. During the night, the PERIOD (PER) and TIMELESS (TIM) proteins accumulate in the cytoplasm and then enter the nucleus to repress their own gene transcription. PER and TIM breakdown during the morning allows repression to be released and a novel cycle to start during the day. Different kinases - DOUBLETIME (DBT)/CK1, CK2, SHAGGY (SGG), phosphatases - PROTEIN PHOSPHATASE 1 (PP1) and PP2A, and ubiquitin ligases - SUPERNUMERARY LIMBS (SLMB), CULLIN-3 (CUL-3) and JETLAG (JET) contribute to PER, TIM and CLK protein modifications, which in turn control their stability, subcellular localization and activity.

The primary function of this “core” *per/tim* feedback loop (Fig. 13) is to regulate protein accumulation, stability, localization and activity in order to set the period of transcript and protein oscillations around 24h.

b. The Clk feedback loop

Interlocked with the *per/tim* feedback loop there is a second loop that controls cycles in *Clk* expression (Fig. 14) (Cyran et al., 2003). Two additional CLK/CYC target genes, *vri* (*vri*) and *PAR domain protein 1ε* (*Pdp1ε*), are activated by CLK/CYC E-box binding at mid-day. VRI and PDP1 are two bZip transcription factors that bind to VRI/PDP1 (V/P) elements on the *Clk* promoter to, respectively, repress and activate transcription of the *Clk* gene, and presumably other downstream targets. As for *per* and *tim*, *vri* mRNA rises at the end of the day and, in phase, VRI protein accumulates in the nucleus at this time. Nuclear VRI binds to V/P elements and represses transcription of the *Clk* gene. Despite being regulated by the same loop, *Pdp1ε* mRNA accumulation is delayed of several hours compared to *vri*, thus PDP1 protein levels peak after mid-night. At this time, VRI levels decline due to decrease in *vri* mRNA and competitive PDP1 binding to V/P elements activates *Clk* transcription at the end of the night/early day. *Pdp1* mRNA and PDP1 protein decrease during the day, and rising *vri* transcription and protein accumulation favors VRI V/P boxes promoter binding and transcriptional inhibition at the end of the day. Transcription of the *cry* gene is similarly regulated.

In a *cyc*⁰ or *Clk*^{Jrk} (severe loss of function mutants) background, CLK/CYC-mediated expression of *vri* and *Pdp1* (as that of *per* and *tim*) target genes is severely reduced but *Clk* mRNA is expressed at constant peak levels (Glossop et al., 1999). This suggests that *Clk* expression is constitutively activated, independently of PDP1ε. Similarly, the constant modification of PDP1ε levels (UAS-*pdp1ε* overexpression or UAS-*pdp1ε*-RNAi downregulation, as well as PDP1ε mutants, *Pdp1*³¹³⁵) exerts little effects on *Clk* mRNA amplitude and waveform (Benito et al., 2007; Zheng et al., 2009). Altogether, data highlighted a prominent circadian role for timed *Clk* transcriptional inhibition by VRI binding rather than timed gene expression through PDP1ε. Altered PDP1ε levels dramatically reduce the locomotor rhythmicity of flies, suggesting that PDP1ε, in addition to play a role in the *Clk* feedback loop, also regulates circadian locomotor behaviors as an output (Benito et al., 2007; Zheng et al., 2009).

In the *per/tim* loop, CLK/CYC-mediated transcription coordinates circadian expression of *vri* (and *Pdp1*) that regulates the *Clk* loop. Nevertheless, the *Clk* loop is not required for the *per/tim* feedback functioning. Indeed, cycles in *Clk* mRNA are independent of CLK protein levels or activity, and expression of *Clk* mRNA in a reversed phase (hence, according to the

per transcription phase) has no effect on the molecular or behavioral rhythms (Kim et al., 2002). Multiple studies on the transcriptome have shown that $\approx 10\%$ of *Drosophila* head mRNAs are rhythmically expressed, and a large fraction of them oscillate with a *Clk*-like phase (peaks near dawn), suggesting a major role for the *Clk* loop in controlling rhythmic output (Ueda et al., 2002; Ceriani et al., 2002; Kula-Eversole et al., 2010; Abruzzi et al., 2011).

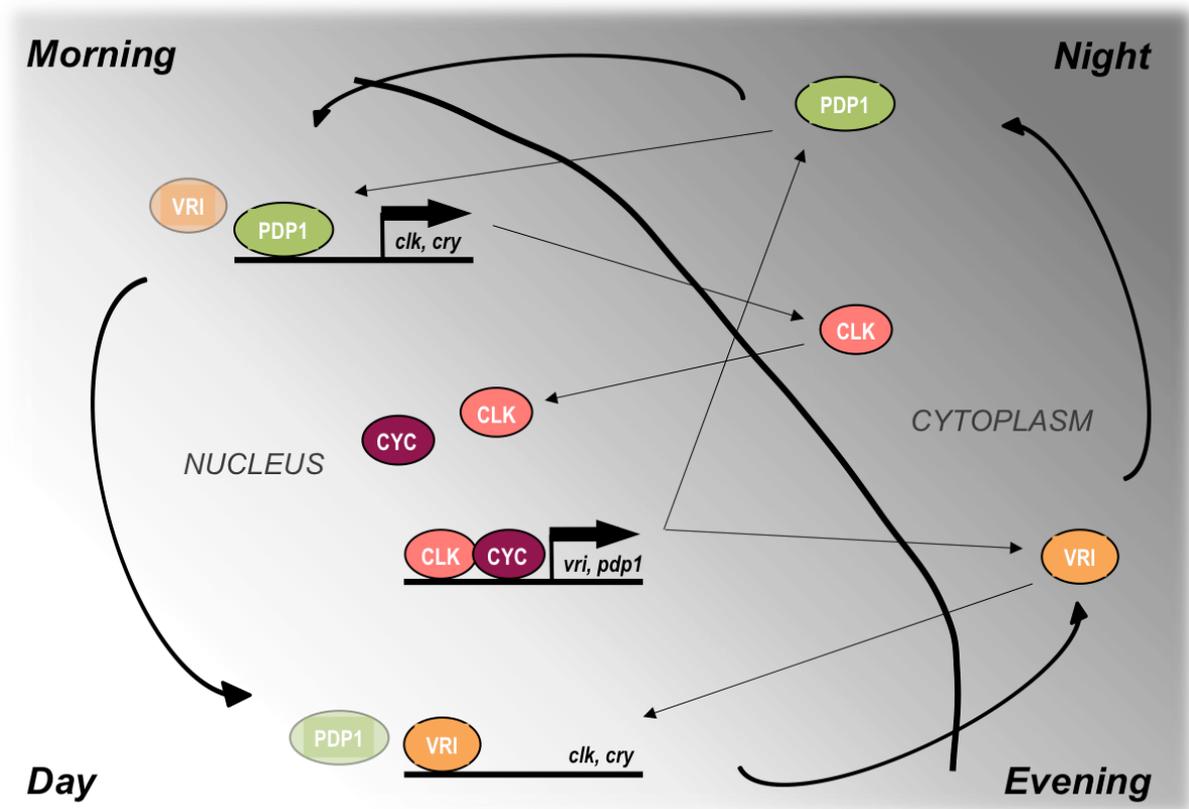


Figure 14 The *Clk* feedback loop

During the day, CLK/CYC binds E-boxes to activate *vri* transcription. As VRI levels rise at the beginning of the night, VRI binds to VRI/PDP1-boxes (V/P-boxes) in the *Clk* promoter, thereby repressing *Clk* transcription. *Pdp1* gene is also transcribed by CLK/CYC transcription factors during the day but *Pdp1* mRNA rises later in the night. PDP1 protein accumulates while VRI protein abundance decreases. PDP1 competes with VRI for binding to V/P-boxes and activate *Clk* transcription at the end of the night - beginning of the day.

Importantly, as a direct consequence of these interlocked feedback loops, *per*, *tim*, *vri* mRNAs oscillate in opposite phase (12h shifted) to *Clk* and *cry* gene expression (Fig. 15).

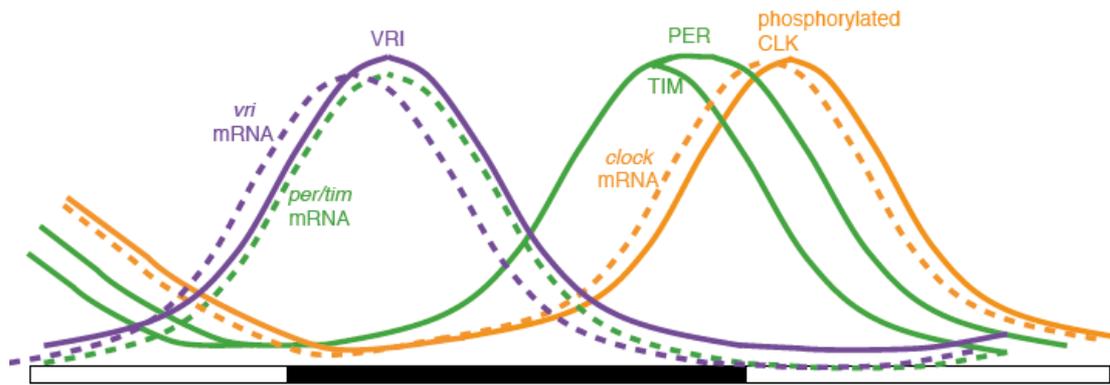


Figure 15 The *per*, *tim*, *vri* and *Clk* mRNAs and their respective protein levels oscillate during a LD cycle. Dotted lines represent mRNA oscillations (violet: *vri*; green: *per* and *tim*; orange: *Clk*), full lines indicate protein oscillations. White and black bars on the bottom show light and dark conditions, respectively.

2. Parallels in other organisms

In all eukaryotes the fundamental basis of circadian rhythm generation is similar (Fig. 16A), yet the players are different, and sometimes even not homologous (Table 1).

a. In fungi: the Neurospora crassa molecular clock

The relatively simple molecular clock in *Neurospora* (Fig. 16C) is composed of two positive regulators: WHITE COLLAR-1 (WC-1) and -2 (WC-2). At night, WC-1 and WC-2 associate to form the WC complex (WCC) and activate transcription of *frq*, which codes for the negative regulator, as well as transcription of a number of clock-controlled genes (*ccgs*). Peak levels of the FRQ protein accumulates with a 4-6 hour delay compared to peak levels of its mRNA. In the morning, FRQ inhibits WCC activity by promoting WCC phosphorylation - a process that also requires CK1a and CK2. This leads to inhibition of *frq* synthesis during the day, thereby closing the feedback loop. However, while inhibiting its activity, FRQ also supports the cytosolic accumulation of WCC, thus acting as a positive regulator for cytoplasmic WCC stability. At night, hyper-phosphorylated FRQ is degraded through the ubiquitin–proteasome pathway, repression on WCC is relieved, WCC is de-phosphorylated and translocates to the nucleus for a novel cycle. (Schafmeier et al., 2006; Gallego and Virshup, 2007) (see also Fig. 22 in § V.3).

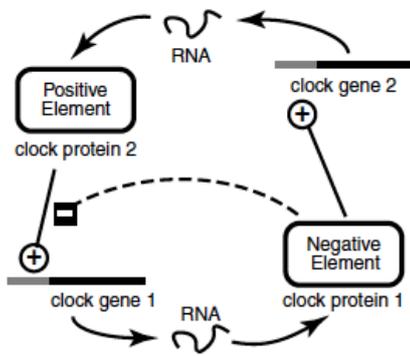
b. The mammalian molecular clock

In the circadian system of mammals, the negative elements of the core feedback loop are PERIOD (PER) 1, 2 and (perhaps) 3 and CRYPTOCHROME (CRY) 1 and 2, while CLOCK and BMAL1 (brain and muscle ARNT-like protein 1) form the heterodimeric transcription factor complex - positive elements (Fig. 16D). In a second loop, REV-ERB α , a member of the retinoic-acid-related orphan nuclear receptors, competes for binding to the ROR elements (RORE) at the promoter region of *bmal1* and regulates CLOCK-BMAL1 activity by inhibiting *bmal1* transcription.

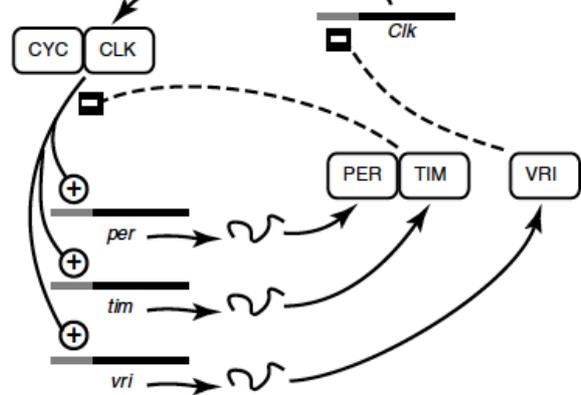
Although most components of the mammalian clock are homologues or functional equivalents of the *Drosophila* circadian clock proteins, some functional re-adaptation or variation can be described. Primarily, the regulation of the mammalian system might be more complex, due to the partial redundancy of the three PER and two CRY paralog isoforms. Further, in contrast to the *Drosophila* system, the main repressors of the mammalian circadian machinery seem to be the CRY isoforms (analogues of the insect TIM protein), rather than PER (nonetheless, constitutive expression of PER2 in the SCN of mice completely abrogate circadian rhythms, while constitutive CRY1 expression do not, questioning for a more critical role of PER, rather than CRY, for circadian oscillation functions - Chen et al., 2009). The *Drosophila* CRY protein functions as a blue-light photoreceptor; conversely, no photoreceptor properties have been demonstrated for mammalian CRY orthologs.

Transcription mechanisms are also quite different. First, while in *Drosophila* the CLK transcriptional partner CYC is constitutively expressed and it is *Clk* that is transcribed and post-translationally modified in a circadian manner, for mammals this oscillating function is rather played by BMAL1 (CYC analogue). Circadian transcription in vertebrates is governed by histone modifications: histone acetylation (that de-condense and “open” chromatin) activates transcription, whereas histone methylation represses transcription (Gallego and Virshup, 2007; Mackey, 2007). In mammals, transcriptional activation is directly linked to the histone acetyltransferase activity of the CLOCK protein itself. Even if this domain is highly conserved among CLOCK homologues in distant species, CLOCK acetyltransferase activity has only been demonstrated in vertebrates. In both *Drosophila* and vertebrates, circadian transcription is regulated by transcription factors’ rhythmic chromatin binding and release (Menet et al., 2010; Rey et al., 2011).

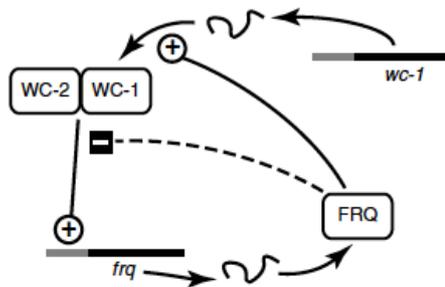
A. Generic Model



B. *Drosophila*



C. *Neurospora*



D. Mammals

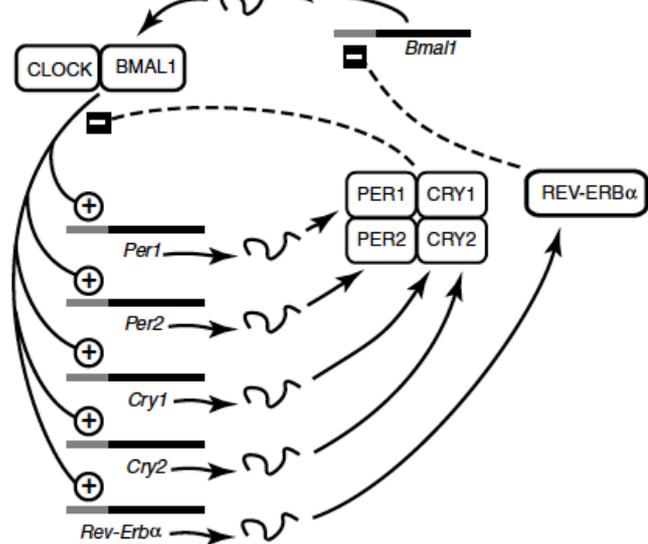


Figure 16 Transcriptional/translational feedback loop (TTFL) models for circadian clocks.

(A) A generic model with two interlocked loops. Clock gene 1 is transcribed into RNA and translated into protein. Clock protein 2 positively regulates transcription of clock gene 1. Clock protein 1 negatively regulates its own transcription by interfering with the positive effects of clock protein 2. Clock protein 1 also positively regulates transcription of protein 2. Based on the generic model, transcriptional/translational oscillator models for *Drosophila* (B), *Neurospora* (C) and mammals (D) are shown (Lakin-Thomas, 2006b).

	Vertebrates	<i>D. melanogaster</i>	<i>N. crassa</i>
Negative factor(s)	Period (PER1, 2 and 3)	Period (PER)	Frequency (FRQ)
	Cryptochrome (CRY 1 and 2)	Timeless (TIM)	
	REV-ERB α	Vrille (VRI)	
Positive factor(s)	Circadian locomotor output cycle kaput (CLOCK)	Clock (CLK)	White collar 2 (WC-2)
	Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (BMAL1)	Cycle (CYC)	White collar 1 (WC-1)
	RAR-related orphan receptor A (RORA)	Par-domain protein 1 (PDP1)	
Kinase(s)	Casein kinase I δ/ϵ (CKI δ/ϵ)	Doubletime (DBT)	CK1-a
	Casein kinase II (CKII)	CK2	CK2
	Glycogen synthase kinase 3 (GSK3)	Shaggy (SGG)	
Phosphatase(s)	Protein phosphatase 1 (PP1)	PP1	PP1
	Protein phosphatase 2A (PP2A)	PP2A	PP2A
Ubiquitin ligase(s)	β -transducin repeat-containing protein 1 and 2 (β -TrCP1 and 2)	Supernumerary limbs (SLMB)	F-box and WD40-repeat-containing protein 1 (FWD1)
	FBXL3	Jetlag (JET)	

Table 1 List of the major components of the molecular feedback loops in vertebrates, and their homologues or analogues in *D. melanogaster* and *N. crassa*. Positive and negative elements of the two interlocked feedback loops are shown. In addition, the most important kinase, phosphatase and ubiquitin ligase enzymes known to function within the clock to modify the previously described positive and negative actors are listed.

c. In prokaryotes: the cyanobacterial clockwork can be reconstituted in vitro

Prokaryotes, which divide multiple times within 24h, were thought to have no need to maintain a timing mechanism that extend farther than their lifetime. However, the ability of sensing external time has helped cyanobacteria to temporally separate incompatible processes such as oxygenic photosynthesis (during the day) and oxygen-sensitive nitrogen fixation (at night) and eventually enhance fitness.

The clockwork in *S. elongatus* is composed of three proteins: KaiA, KaiB and KaiC (Fig. 17A), whose mRNAs accumulate in a circadian manner. KaiA and KaiC are, respectively, the positive and negative regulators of the *kaiBC* dicistronic gene expression (Mackey and Golden, 2007).

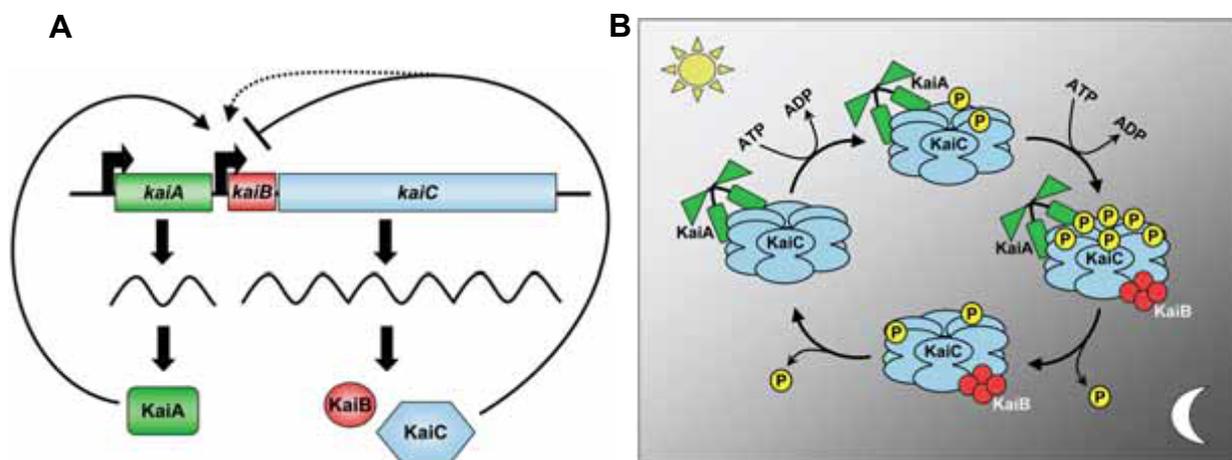


Figure 17 The oscillator in *S. elongatus*

A The KaiABC transcriptional oscillator. The *kaiBC* is a dicistronic gene which expression depends on a promoter located upstream the *kaiB* ORF. KaiA acts as a positive regulator for *kaiBC* gene transcription, while KaiC feeds back to repress its own transcription. A basal level of KaiC protein is required for *kaiBC* expression (dotted line). **B** The post-translational KaiABC oscillator. During the day, KaiA associates with KaiC hexamers and stimulate the auto-phosphorylation of KaiC. Hyper-phosphorylated KaiC undergoes conformational changes that allow KaiB to bind: this results in dephosphorylation of KaiC. Hypo-phosphorylated KaiC releases KaiA and KaiB, allowing another cycle to begin (Mackey and Golden, 2007).

Although *kaiA* and *kaiBC* mRNA cycles, levels of the KaiA protein are constant and the KaiB and KaiC protein levels peak few hours after their mRNA highest level, suggesting the existence of post-translational regulation (Fig. 16B). Indeed, KaiC phosphorylation varies in a circadian manner. KaiA enhances auto-kinase activity of KaiC and stimulates KaiC hyper-phosphorylation. Later, KaiB binding to the KaiA/C complex antagonizes KaiA and promotes

KaiC auto-phosphatase activity, which returns KaiC to a hypo-phosphorylated state (Johnson et al., 2011).

Intriguingly, the *KaiABC* molecular oscillator was reconstituted in a test tube, just putting together the three Kai proteins and ATP (Nakajima et al., 2005). This *in vitro* oscillator ticked with a ~24h period for at least 10 days and possessed properties of being temperature-compensated. During the 24h oscillation, the KaiC protein underwent rhythmic changes in conformation, phosphorylation status and interaction with the KaiA and KaiB proteins. By definition, these changes were solely mediated by post-translational (phosphorylation) modifications (Johnson et al., 2011).

3. Circadian transcription is not necessary for the core clock functions

Similarly to the *Synechococcus* clock functioning, evidence exists that even the eukaryotic clockwork can operate without transcriptional cycles.

Mice null for *rev-Erba* (the repressor of the *bm11* gene transcription) are rhythmic in DD and constant light (LL) conditions (Lakin-Thomas, 2006b), indicating that rhythmic *bm11* expression is not required for functional BMAL1 transcriptional activities. Similarly in *Drosophila*, forced constitutive expression of the transcription factor CLK, or its expression 12h later (in anti-phase) still support normal clock functions (Kim et al., 2002). Moreover, flies in which the transcriptional repressor *per* is transcribed by a constitutive, rather than a rhythmic, promoter still show normal circadian activity rhythms, even if weaker ones (Yang and Sehgal, 2001). Likewise, mammalian *per1* and *per2* can be expressed in a constitutive manner without affecting circadian rhythmic functions. However, these observations still do not disprove that cycles in the transcription of some other clock genes will be indispensable.

Strong insights into the existence of biological non-transcriptional oscillators have recently come. First in liver cells (Reddy et al., 2006) and also in human hemoglobin, peroxiredoxin (PRX) was shown to undergo 24h rhythms in oxidation, which persisted for many days under constant conditions. These cycles could be entrained by temperature and showed temperature-compensation properties, thus matching all criteria for circadian rhythms. *Ergo*, a-nucleate human red blood cells could sustain circadian biochemical rhythms even in the absence of gene expression (O'Neill and Reddy, 2011). Similar rhythms in PRX oxidation were also found in cyanobacteria, algae (*Ostreococcus tauri*), *Neurospora*, fruit flies and mice (O'Neill

et al., 2011). Therefore, bacteria, plants, fungi and animals do share a common timekeeping mechanism based on metabolic (oxidative) cellular state (*Metaclock*).

Nevertheless, it is worthy to note that rhythmic transcription may hold other essential functions for the circadian system. Circadian transcription of clock-controlled genes strongly participates, directly or indirectly, in driving timed, rhythmic, behavioral and physiological “output” pathways. The transcriptional control increases the amplitude of protein cycles, thus contributing to the maintenance of robust oscillations and helping to buffer fluctuations of environmental cues (Lakin-Thomas, 2006a; Kadener et al., 2008; Edery, 2011).

4. Post-transcriptional and post-translational regulation of clock proteins

Post-transcriptional and post-translational modifications are crucial to create a defined delay between transcriptional activation and transcriptional repression that avoids generation of a steady state and allows the production of a circuit that takes approximately 24 hours (Harms et al., 2004) (Fig. 18). The time of gene transcription, RNA processing (mainly by alternative splicing) and (weak) mRNA stability orchestrates a timed availability of proteins. Dynamic changes in phosphorylation regulate kinetics of protein turnover, their subcellular distribution and their activity.

Remarkably, the existence of these diverse steps of regulation multiplies the ways to sense external changes (input) and coordinates the production of a stronger response (output).

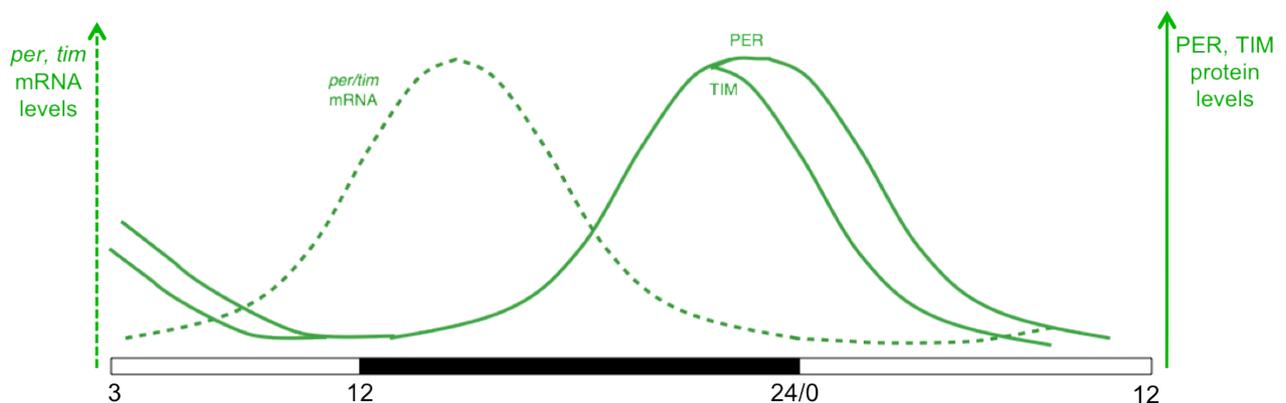


Figure 18 Time switch between the oscillations of *per* and *tim* mRNAs (dotted line) and PER and TIM proteins (full line). Post-transcriptional and post-translational mechanisms control times of protein accumulation and turnover. White and black boxes indicate light and dark conditions, respectively.

a. Post-transcriptional modifications

For transcripts to be rhythmic and with high amplitude oscillations they are required to have a relatively short half-life (Kojima et al., 2011). Post-transcriptional modifications have been shown to affect mRNA stability and translational rates of clock genes in many species (*Neurospora*, *Drosophila*, mice).

In *Drosophila*, expression of a *per* transgene via a promoter-less construct allows *per* mRNA cycling with an amplitude low but sufficient to rescue arrhythmic behavior of *per*-null mutants (Yang and Sehgal, 2001), suggesting the existence of post-transcriptional changes that affect stability of the *per* transcripts. This regulation depends on a region located in the first intron of *per* (Stanewsky et al., 2002) that affects mRNA stability and rate of translation. As 3' splicing may be controlled by light and temperature, this mechanism allows seasonal sensing. Similarly to *per*, a naturally occurring *tim* polymorphism (*tim-ls*) produces two *tim* transcripts (*tim-s* and *tim-l*): homozygous *tim-ls* flies are less sensitive to light-mediated TIM degradation (Peschel et al., 2006; Peschel et al., 2009). *Drosophila per* translation is also regulated. Very recently, two proteins - TWENTYFOUR (TYF) and ATAXIN-2 (ATX2) were described that bind to the poly-A region of the *per* mRNA and cooperate to activate its translation (Lim et al., 2011; Lim and Allada, 2013; Zhang et al., 2013). Translational regulation also occurs through microRNAs. In the fruit fly, the microRNA *bantam* targets 3'-UTR sequences of *Clk* mRNA (Kadener et al., 2009); microRNA-mRNA pairing have also been shown for *cwo* and *vri* transcripts.

b. Post-translational modifications: phosphorylation controls protein stability, localization and activity

Phosphorylation is a post-translational modification widely used by cells to regulate, activate or modify various cellular processes, including circadian cycles. Phosphorylation can change the activity of a protein (i.e. kinases), its binding properties, its subcellular localization and its stability; hence, phosphorylation regulates the general functions of a protein (Tataroglu and Schafmeier, 2010).

In all studied circadian clocks, both the positive and negative elements were shown to undergo daily cycling in phosphorylation. The phase-specific import (and export) of clock components into (and from) the nucleus is of great importance to impose accuracy on the period length. Studies in cultured *Drosophila* cells showed that intermediate phosphorylation

of CLK in the cytoplasm supports CLK/CYC nuclear translocation (Maurer et al., 2009), whereas hyper-phosphorylation is accompanied by CLK nuclear export (Hung et al., 2009). In addition, phosphorylation promotes the nuclear entry of the PER/TIM/DBT repressor complex. TIM phosphorylation by the GSK3 orthologue SGG kinase is a prerequisite for nuclear import of the repressor complex (Martinek et al., 2001). Similarly, the cytoplasmic activity of the CK2 kinase modulates the timing of nuclear import of PER in *Drosophila* neurons (Lin et al., 2002; Akten et al., 2003). Phosphorylation was shown to regulate changes in the subcellular localization of all the major clock components in different species (Fig. 19) (Reischl and Kramer, 2011; Tataroglu and Schafmeier, 2010).

Phosphorylation also correlates with the activity of both the positive and negative element(s) in a variety of species. Hyper-phosphorylated forms of the *Drosophila* CLK protein accumulate at times of minimal transcription rates (§ V.2.c). Furthermore, *Drosophila* PER repressor activity is enhanced by DBT and CK2 kinases (Nawathean and Rosbash, 2004).

Importantly, phosphorylation controls the stability, thus the quantity levels of proteins, in particular the repressor(s). Phosphorylation is required for the recruitment of most (even if not all) ubiquitin ligases, which mediate the poly-ubiquitylation and the subsequent degradation of proteins by the proteasome (Gallego and Virshup, 2007) (§ IV.4.c). In *Drosophila*, phosphorylation by the DBT kinase during the late day degrades newly synthesized PER and delays PER protein accumulation (Price et al., 1998). Similarly, hyper-phosphorylated PER at the end of the cycle (during the day) is degraded in a proteasome-dependent manner. PER phosphorylation likely occurs in clusters and it is sequential in such phosphorylation at certain sites requires priming phosphorylation at other sites (Chiu et al., 2008; Chiu et al., 2011). DBT activities also affect stability of the CLK protein (§ V.1.c). Reduced CK2 activity (*Timekeeper (Tik)* and *Andante (And)* mutations) promotes both PER and TIM stabilization, but also negatively affects stability of the CLK protein (Szabo et al., 2013).

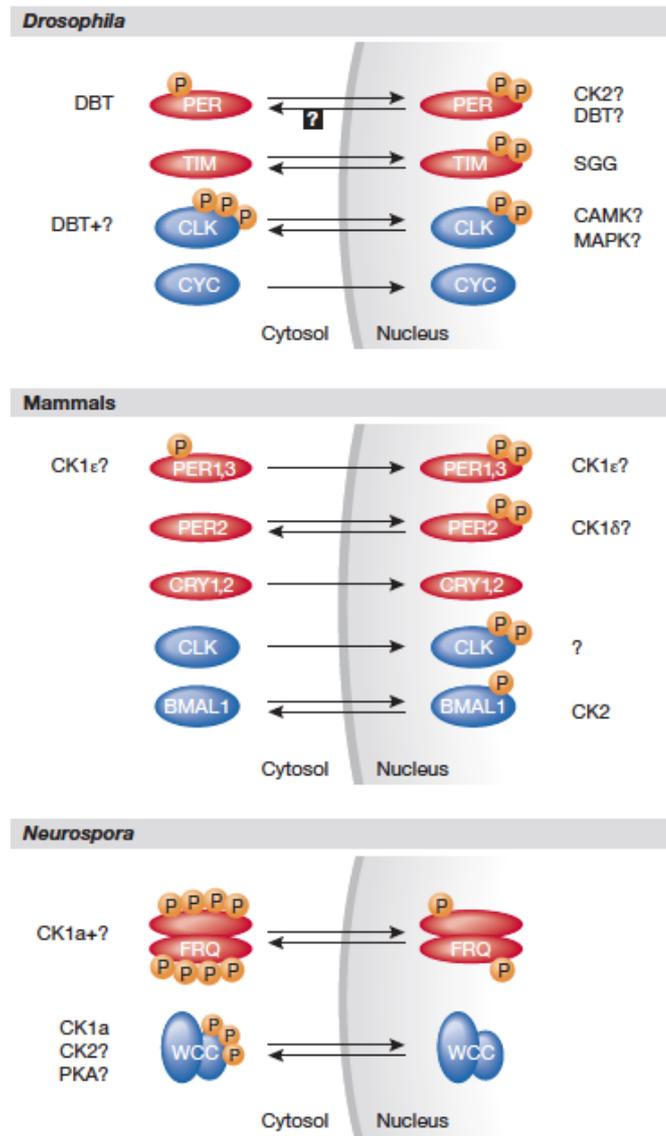


Figure 19 Phosphorylation affects the subcellular localization of clock proteins.

The figure recapitulates the subcellular trafficking of clock activators (blue) and repressor (red) proteins within the *Drosophila*, mammalian and *Neurospora* molecular clocks. The kinases involved in modifying protein localization are listed (Tataroglu and Schafmeier, 2010).

c. Post-translational modifications: ubiquitylation (and proteasomal degradation)

Throughout the phyla, an essential feature of the clock is the robust daily oscillations in the quantities of the repressor protein - vertebrate and *Drosophila* PER, CRY and TIM, and FRQ in *Neurospora*. All of these proteins are degraded through phosphorylation-regulated, ubiquitin-directed, proteasome-mediated proteolysis (Fig. 20).

Ubiquitylation (i.e. ubiquitin conjugation) of targeted substrates is catalyzed by the sequential action of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes. Thanks to their high number (over 300 in *Drosophila*), the E3 ubiquitin-ligase enzymes carry the specificity of the reaction.

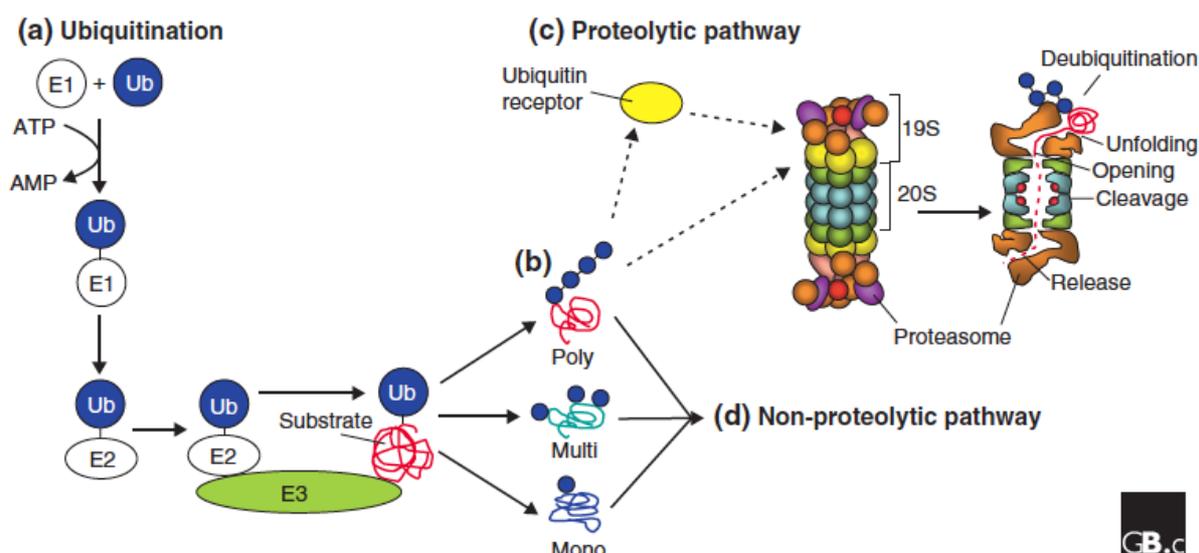


Figure 20 Mechanisms of protein ubiquitylation and the ubiquitin-proteasome system.

(a) Ubiquitin is transferred to a targeted substrate by subsequent activities of an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligases (E3). **(b)** The substrate can be targeted by addition of a single ubiquitin molecule (mono-ubiquitylation), multiple ubiquitin units at different Lys residues (multi-ubiquitylation) or polymerized ubiquitin chains (poly-ubiquitylation). **(c)** Many poly-ubiquitylated proteins are degraded by the 26S proteasome, which is composed by a catalytic 20S complex and a regulatory 19S unit. Ubiquitylated protein can be directly bound by the ubiquitin-binding activity of the 19S proteasome or are addressed to the proteasome through soluble ubiquitin receptors. The 19S subunit recognizes the ubiquitylated substrate and promotes disassembling of the ubiquitin chain; the substrate undergoes unfolding and enters the cavity of the 20S unit, where proteolysis takes place. **(d)** Poly-, multi- or mono-ubiquitylation also regulates protein functions in a proteolysis-independent manner (Kaiser and Luang, 2005).

In *Drosophila*, PER phosphorylation by DBT on Ser47 is considered the “death-kiss” for recognition by an F-box protein/Cullin1 ubiquitin ligase containing SUPERNUMERARY LIMBS (SCF^{SLMB}) that polyubiquitylates PER and targets it for proteasomal degradation (Grima et al., 2002; Ko et al., 2002). The TIM protein is also degraded after phosphorylation-dependent SLMB recognition and targeting, which regulates TIM proteasomal degradation in constant darkness conditions (Grima et al., 2002). Importantly, SLMB may participate to TIM degradation in the nucleus while a BTB-domain protein/Cullin3 ubiquitin ligase enzyme (CUL3) seems important for TIM degradation in the cytoplasm in free-running conditions (Grima et al., 2012). TIM also undergoes a light-mediated proteasomal breakdown. This process involves another F-box protein/Cullin1 ubiquitin ligase, SCF^{JETLAG(JET)}, which targets both TIM and (possibly) the light-activated TIM partner CRYPTOCHROME (CRY) proteins (Koh et al., 2006; Peschel et al., 2006; Peschel et al., 2009). Ubiquitylation regulates the stability of the *Drosophila* transcription factor CLK (Lamaze et al., 2011) and also its activity (see § V.2.c).

Importantly, ubiquitylation can be reversed by deubiquitinating enzyme (DUB) proteases: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (USPs/UBPs). DUBs hydrolyze the isopeptide bond between ubiquitin and target proteins, and between adjacent ubiquitins in poly-ubiquitin chains, thus recycling free ubiquitin. Importantly, the disassembly of free poly-ubiquitin chains is essential to avoid their accumulation, which can inhibit the proteasome (Amerik et al., 1997). Isopeptidase T (IsoT)/Usp5, homolog of *S. cerevisiae* UBP14, is responsible for the disassembly of the majority of unanchored poly-ubiquitin chains in mammals (Dayal et al., 2009; Amerik and Hochstrasser, 2004).

d. Post-translational modifications: dephosphorylation

The net phosphorylation state of every phosphoprotein depends on a balance between kinase and phosphatase activities (Gallego and Virshup, 2005). As previously discussed, phosphorylation controls activity, localization and stability of circadian proteins. Although precisely regulated, kinases are widely expressed in the cell and their quantity does not oscillate within a day-length. To accomplish their circadian role, kinase activities must be controlled in time- and in a cell compartment-specific manner. Time- and space-regulated dephosphorylation events are thus thought to counteract kinase activity in a cycle-specific

moment. With that, phosphatases, rather than kinases, account for the circadian control of protein phosphorylation (Sathyanarayanan et al., 2004).

The most extensive studies on the role of protein phosphatases in the circadian clock were conducted on the bread mold *N. crassa*. In *Neurospora*, PP1-mediated dephosphorylation promotes the stabilization of FRQ, the repressor element. In addition, PP2A controls dephosphorylation and thus reactivation of WCC (Fig. 21), which occurs exclusively in the cytoplasm (Schafmeier et al., 2008) and correlates with decrease in FRQ levels.

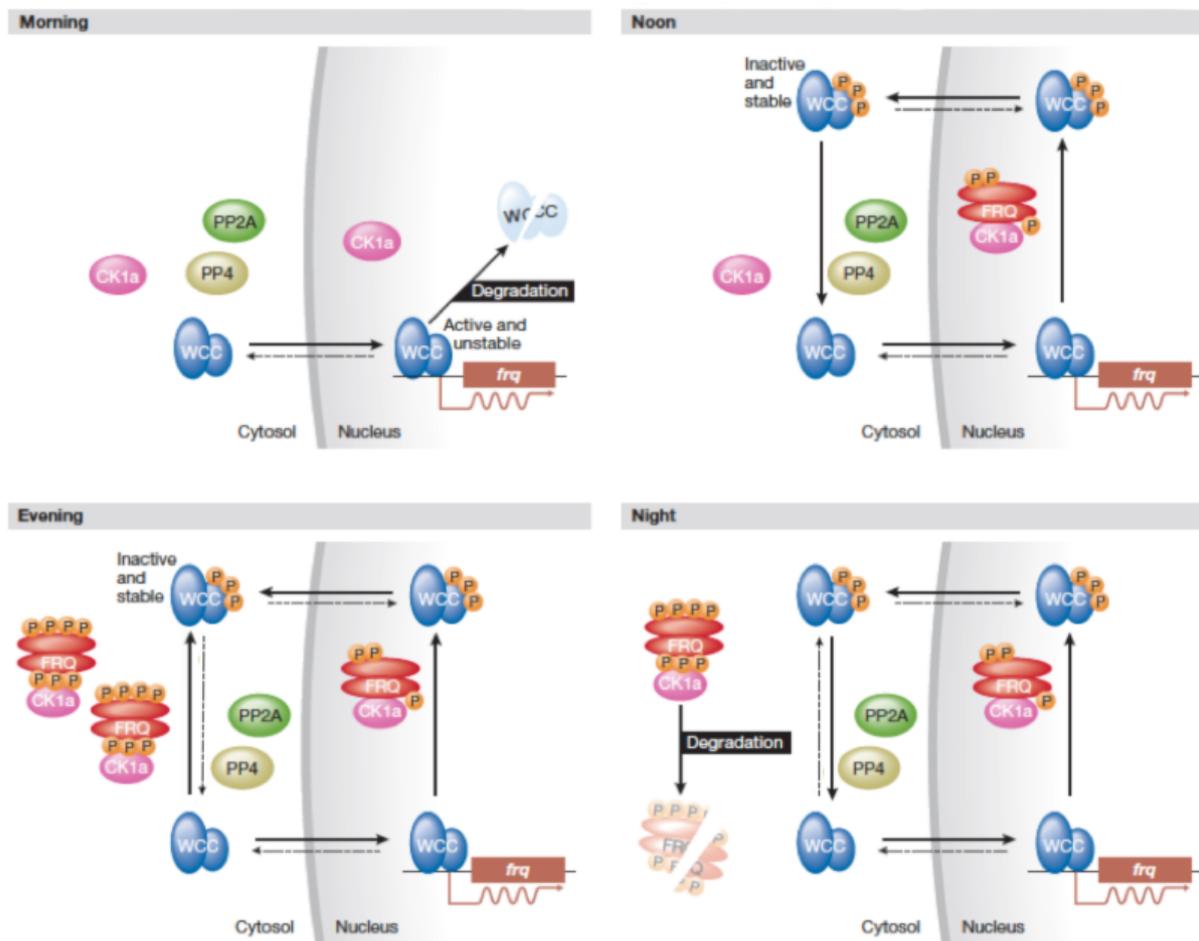


Figure 21 Modulation of WCC shuttling and activity by phosphorylation and dephosphorylation.

In the morning, un-phosphorylated and active WCC enters the nucleus and transcribes targeted genes, like *frq*. Active WCC is unstable and rapidly degraded. FRQ accumulation during the day allows FRQ-mediated WCC phosphorylation and stabilization in an inactive form. FRQ degradation at night tilts the balance towards WCC dephosphorylation by PP2A and PP4: activated WCC re-enter the nucleus for a novel cycle. (Tataroglu and Schafmeier, 2010)

The *Drosophila* circadian clock is sensitive to variation in the PP2A phosphatase activities. Broad (in *tim*⁺ cells) overexpression of wild-type *mts* (PP2A catalytic subunit) as well as a dominant-negative form of *mts* (*mts*^{DN}) renders flies arrhythmic (Sathyanarayanan et al., 2004). Two PP2A regulatory subunits (see § VIII.1.b) were shown to mediate PP2A roles in the fruit fly clock. RNA interference of either *tws* or *wdb* regulatory subunits induces PER (Sathyanarayanan et al., 2004) and CLK (Kim and Edery, 2006) protein degradation in *Drosophila* S2 cell culture. Interestingly, both *wdb* and *tws* genes are expressed in a circadian fashion, but in opposite phases: *wdb* mRNA peaks at the end of the night/beginning of the day (as *Clk* mRNA), while *tws* mRNA accumulates at the end of the day and diminishes during the night (as *per* mRNA). Yet, no data are available concerning a cycle in TWS and WDB proteins quantity. TWS overexpression in *pdf*⁺ cells induces slightly shorter periods (23,2h/24,4h; R=70%) while WDB overexpression in *tim*⁺ cells lengthens the locomotor periods (25,5/23,5) (Sathyanarayanan et al., 2004). Data suggest that the two regulatory subunits differently affect the clock machinery.

Immunohistochemistry data from larvae and western blot analysis of adult head extracts overexpressing wild-type or mutant *mts* suggest that PP2A functions to promote PER nuclear localization and to control PER stability. PP2A counterbalances PER phosphorylation by CKIδ (which is 93% similar to *Drosophila* CKIε/DBT) *in vitro* (Sathyanarayanan et al., 2004). Altogether, data suggest that PP2A counteracts the DBT-mediated PER phosphorylation that induces PER degradation (Fig.22).

PP1 is also relevant for the clock functions of the fruit fly (Fang et al., 2007). Overexpression of a nuclear inhibitor of PP1 (NIPP1) lengthens the period of locomotor rhythmicity. *in vitro* phosphorylated TIM is a direct substrate for purified PP1, and TIM co-immunoprecipitates with the PP1 catalytic subunit in cultured cells. TIM is destabilized upon PP1 inhibition in both S2 cells and in larval lateral clock neurons, while TIM oscillates with lower amplitude, compared to controls, in Western blots. These effects persist in a *per* null background, suggesting that PP1 stabilizes TIM independently of the feedback loop and its partner PER. However, PER is also destabilized after PP1 inhibition but effects on PER are secondary to those on TIM. Data are consistent with PP1 stabilizing TIM, which promotes accumulation of PER.

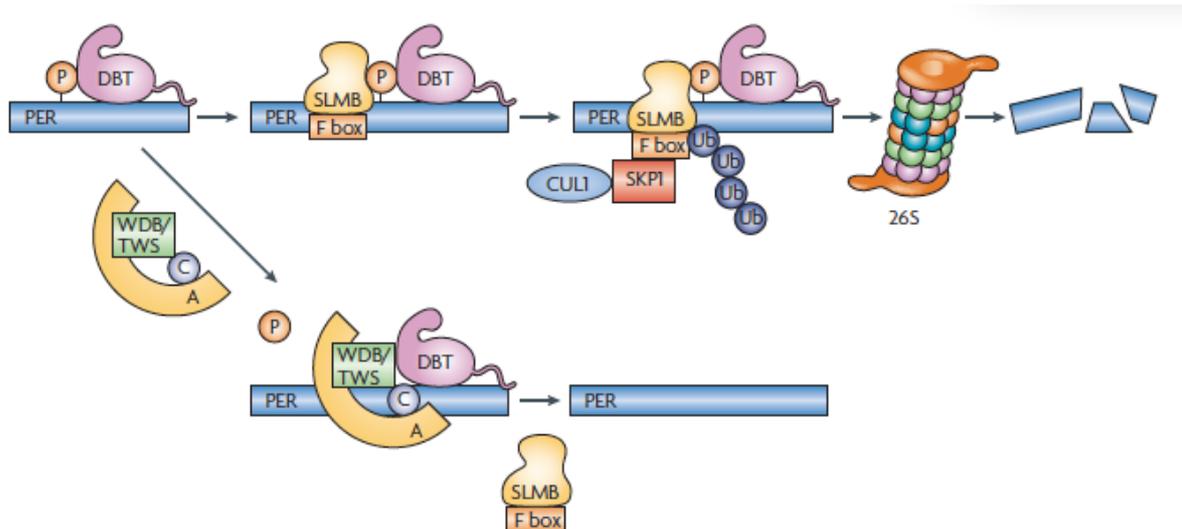


Figure 22 PP2A counteracts DBT-dependent PER phosphorylation that triggers PER degradation.

PER phosphorylation by DBT allows PER recognition by the SCF^{SLMB} ubiquitin ligase, which targets PER for degradation by the proteasome. PP2A balances phosphorylation events on PER, thus promoting its stability. PER recognition by the PP2A complex occurs through either the WDB or the TWS PP2A regulatory subunits. (Gallego and Virshup, 2007)

As previously discussed, PP2A plays a key role in the dephosphorylation events that modulate the activity and stability of both the repressor (PER) and the activator (CLK) components of the *Drosophila* circadian machinery. A special chapter of this manuscript (§ VI) will be dedicated to the mechanisms of PP2A holoenzyme assembly, substrate specificity and regulation of its activity within PP2A-containing super-complex.

V. A FOCUS ON THE REGULATION AND ACTIVITY OF THE *Drosophila* CLK PROTEIN

During the past years, multiple studies have highlighted more and more in depth the mechanisms that control PER and TIM protein abundance, cytoplasm-nucleus shuttling and repressive activity on circadian transcription. Much less is known about the mechanisms that control CLK protein life cycle, its stability and its activity. Here is a chapter focusing on all we know about the post-translational regulation of the CLK protein and the mechanisms that govern CLK/CYC-mediated transcription of circadian-controlled genes.

1. Regulation of the *Drosophila* CLK protein

a. CLK is present in limiting amounts and constantly binds CYC

The CLK protein is present in fly heads in limiting amounts: peak of its molar concentration has been shown to be 4 to 6-fold lower than the highest value obtained for PER and TIM, and around 200-fold lower than CYC protein quantity (Bae et al., 2000). Nevertheless, CLK can activate transcription of target genes to relatively high amounts, which makes CLK a very potent activator (Yu et al., 2006; Menet et al., 2010; S. Kadener – personal communication).

Reciprocal coimmunoprecipitation assays reveals that likely all the CLK protein interacts with CYC throughout a daily cycle (Bae et al., 2000). CYC is constitutively expressed both in fly heads and in cultured *Drosophila* Schneider2 (S2) cells. The S2R+ cultured line, which is derived from S2 cells, possesses half of *cyc* mRNA and protein normally present in the S2 cell line. When transfected with a CLK plasmid, the CLK protein accumulates mainly in the cytoplasm in S2R+ cells. Exogenous supplement of CYC promotes CLK nuclear staining, which is the common localization seen for CLK in S2 cells and in brain neurons. This indicates that CYC, *in vitro* and presumably *in vivo*, promotes nuclear translocation of the CLK/CYC heterodimer (Hung et al., 2009).

b. CLK quantities are tightly regulated

Immunocytochemistry on whole-mounted wild-type fly heads shows comparable CLK protein amounts and constant CLK nuclear staining in oscillator cells throughout a daily cycle (Houl et al., 2006). On the other hand, CLK protein analysis by Western blot shows circadian variations in its molecular weight profile (post-translational modifications, mainly phosphorylation) and possibly in CLK quantities. The *Clk* mRNA peaks at the end of the night/beginning of the day and the CLK protein is thought to accumulate in phase with its messenger (Fig. 23 – orange lines), as in Western blots much CLK protein is seen at the beginning of the day and lower CLK levels are present at night. Yet, harsh CLK protein extraction using more stringent procedures (sonication) likely allows release of chromatin-bound CLK, which results in CLK quantities remaining globally constant throughout a daily cycle (Yu et al., 2006) (Fig. 23 - red full line).

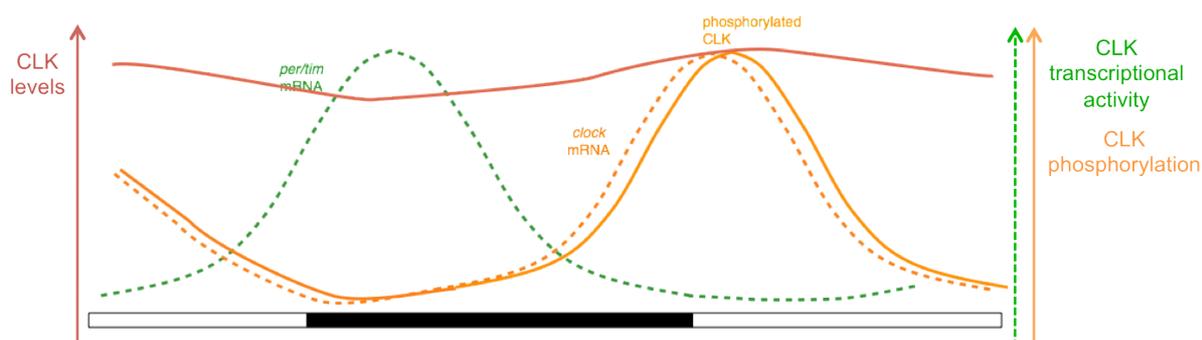


Figure 23 Oscillations in the CLK protein quantity, phosphorylation and activity.

Clk mRNA (orange dotted line) oscillates with a peak at the beginning of the day and a bottom during night. However, the overall CLK protein levels (red full line) appear to remain globally constant during the cycle. Yet, the CLK protein undergoes cycles in phosphorylation (orange full line) that follows cycles in mRNA accumulation. Remarkably, maximal CLK-mediated transcription (*per/tim* mRNA – green dotted line) occurs at times when CLK is low phosphorylated. White and black boxes indicate the time of lights-on and lights-off, respectively.

ARK is a fly line expressing a HA-tagged *Clk* cDNA transgene regulated by a *per* promoter, that drives *Clk-HA* transcription in the opposite circadian phase compared to wild-type endogenous *Clk* mRNA. Those flies display normal behavioral rhythms and a wild-type CLK-HA profile on Western blots, which supports a prominent role of post-translational modification in controlling CLK amounts and timed CLK phosphorylation (Kim et al., 2002;

Kim and Edery, 2006). However, CLK-HA is slightly more abundant, compared to wild-type extracts, at the beginning of the night, *in sync* with transcription from the *per* promoter region, and consistent with hypo-phosphorylated CLK being more stable. Hyper-phosphorylated CLK, found *in vivo* during late night and day, is unstable (Yu et al., 2006; Weber, 2009). In S2 cells, hyper-phosphorylated ones are the first CLK forms that disappear upon DBT induction; these phospho-forms are even the most stabilized upon MG132 proteasome inhibitor treatment. Thus, CLK likely undergoes proteasome-mediated degradation *in vitro* and possibly *in vivo* (Kim and Edery, 2006). The E3 ubiquitin-ligase CTRIP (Lamaze et al., 2011) has been proposed to target CLK for proteasome recognition.

The KAYAK (KAY) bZip transcription factor indirectly regulates *Clk* mRNA quantities (Ling et al., 2012). KAY interacts with VRI and limits VRI-mediated repression of *Clk* gene transcription (Fig. 24). In addition, KAY also acts to inhibit CLK target gene transcription, likely through promoting the expression of a CLK/CYC repressor.

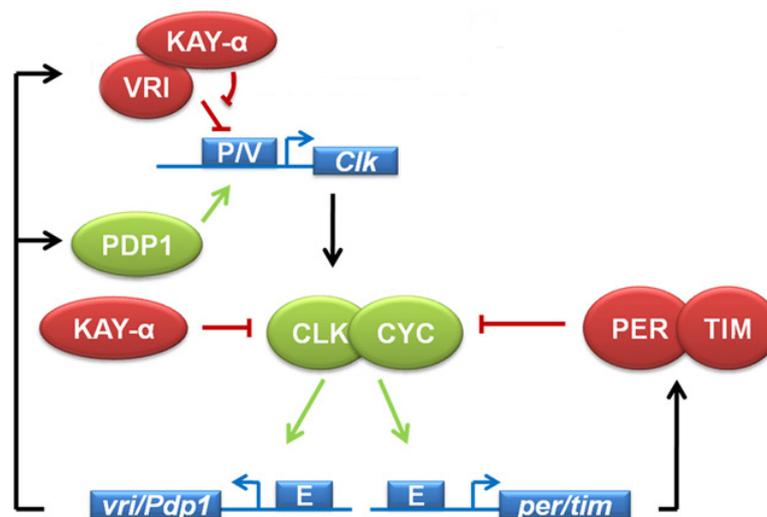


Figure 24 KAYAK (KAY) plays a dual role within the circadian oscillator of *Drosophila*.

KAY α inhibits VRI through directly binding and forms a VRI/KAY α dimer no more able to bind to V/P elements on the *Clk* promoter. IN addition, KAY α represses CLK transactivation (Ling, 2012).

CLK quantities are tightly controlled also at the post-transcriptional level. Freshly transcribed versus steady state comparison of endogenous *Clk* mRNA shows that *Clk* mRNA is very unstable. Possibly, this is due to the fact that *Clk* mRNA possesses a short poly-A tail (S. Kadener - personal communication). miRNA, like *bantam* (Kadener et al., 2009) have also been proposed to control *Clk* mRNA stability.

c. DBT affects CLK phosphorylation and stability within a DBT/PER/CLK complex

In *per*⁰ or *cyc*⁰ flies, where no PER protein is present, rhythms in CLK phosphorylation are completely abolished and CLK persists as a primarily hypo-phosphorylated variant (even though it migrates more slowly than wild-type hypo-phosphorylated CLK, suggesting some extent of CLK phosphorylation that are not dependent on PER) (Yu et al., 2006). Indeed, CLK associates with PER/DBT-containing complexes at the beginning of the day, and formation of CLK-DBT complexes depends on PER (Yu et al., 2006).

DBT was initially proposed as a possible CLK kinase. Indeed, DBT is required for proper CLK phosphorylation. A *per* mutant unable to bind DBT (*perΔ* flies) eliminates CLK hyper-phosphorylation (Kim et al., 2007). Moreover, DBT overexpression in S2 cells induces an upward (low migrating) shift in CLK staining profile on blots. Importantly, these effects are independent of CLK's ability to bind chromatin (*Clk^{ADBD}*) or its partner CYC (Kim and Edery, 2006).

However, the role of DBT as a direct CLK kinase is questionable. In *dbt* hypomorphic (*dbt^P/dbt^{aw}*) mutant flies (as well as in *dbt* dsRNA-treated S2 cells) CLK accumulates in a “medium-phosphorylated” variant (i.e. in-between the hypo- and hyper-phosphorylated forms) and loses molecular oscillations (Yu et al., 2006; Yu et al., 2009). Yet, hyper-phosphorylated CLK forms are missed. The loss of hyper-phosphorylated CLK variants in those mutants may nevertheless be the consequence of either low DBT quantities (that cannot assure normal CLK phosphorylation as less DBT-containing CLK complexes occurs in mutants) or impaired DBT kinase activity. *dbt^{K/R}* is a *dbt* allele bearing a single amino-acid substitution (Lys69Arg, K69R) in the DBT polypeptide chain that produces a correctly folded DBT protein, which in contrast lacks any biochemical activity. Overexpression of a *dbt^{K/R}* transgene using the UAS-*Gal4* technique efficiently titrate endogenous wild-type DBT activity in the Gal4 expressing cells (dominant-negative allele), while it prevents embryonic death caused by loss-of-function *dbt* alleles (Muskus et al., 2007). DBT^{K/R} overexpressing flies still show non-cycling hyper-phosphorylated CLK. Therefore, DBT is not necessary for the majority of CLK phosphorylation (at least low to medium-phosphorylated variants) – which is therefore accomplished by other kinase(s), but, importantly, DBT is required for phase-specific CLK hyper-phosphorylation (Yu et al., 2009). In addition, these experiments highlight the importance of DBT physical occupancy, instead of DBT kinase activity, for its effects on the CLK protein.

DBT also regulate CLK stability. *dbt* dsRNA treatment in S2 cells stabilizes CLK; similarly, increased CLK protein amounts are seen in *dbt* hypomorphic mutants (*dbt^P/dbt^{ar}*), suggesting a role for DBT in destabilizing CLK. However, in contrast to other *dbt* mutants, the hyper-phosphorylated CLK present in DBT^{K/R} overexpressing flies fails to accumulate and rather shows less prominent staining compared to controls (Yu et al., 2009). Consistently, increasing of the DBT amounts (transfection of *dbt*-containing plasmids) in S2 cells shows that at very high DBT concentrations, the balance tilts toward dephosphorylation and stabilization of CLK (Kim and Edery, 2006). Therefore, the molecular profile of the CLK protein seen on Westerns is exquisitely sensitive to DBT quantities.

d. CLK phosphorylation requires still undefined kinase(s)

As previously discussed, the kinase(s) responsible for the majority of CLK phosphorylation has not been identified yet.

Activation of CLK/CYC heterodimer in S2 cells is affected by calcium-calmodulin-dependent kinase II (CaMKII) and mitogen-activated protein kinase (MAPK), which both can phosphorylate CLK *in vitro* (Weber et al., 2006).

in vivo, the NEMO (NMO) kinase has been implicated in CLK phosphorylation (Yu et al., 2011). Decreasing NMO functions increases CLK levels and phosphorylation and induces short period behaviors. Conversely, CLK is destabilized in *nmo* overexpressing flies. Studies from Edery and colleagues describe an interesting role for NMO-mediated phosphorylation in gating DBT-dependent PER phosphorylation and degradation (Chiu et al., 2011). As times of PER hyper-phosphorylation correlates with times when CLK is unstable, whether NMO lowers CLK levels by directly phosphorylating CLK or, indirectly, by phosphorylating PER is thus questioned. Nevertheless, NMO-CLK complexes are found at all times during the cycle, supporting a role for NMO in CLK phosphorylation events which is independent of PER.

e. Sequential and compartment-specific phosphorylation controls CLK life cycle

Analysis of the CLK amino acid sequence (Hung et al., 2009) combined with data from cultured S2 cells experiments reveals the presence of two nuclear localization signals (NLS), one of which is unique to *Drosophila* CLK protein and is necessary for the majority of CLK

nuclear translocation (even if some nuclear CLK staining can be seen in CLK^{ANLS}-expressing cells); and one nuclear exporting signal (NES), the deletion of which (CLK^{ANES}) results in a complete nuclear CLK localization.

In cultured S2 cells, transfection of *Clk*^{ANLS} induces a strong hypo-phosphorylated cytoplasmic CLK staining, while medium- to hyper-phosphorylated CLK is present to lower levels in the nucleus of CLK^{ANES} mutants. Hence, nuclear translocation is required for CLK phosphorylation to occur. However, Western analysis of extracts from S2 cells transfected with a wild-type *Clk* shows an even less migrating, hyper-phosphorylated CLK band compared to extracts from cells transfected with *Clk*^{ANES} plasmids. Hence, nuclear export enhances CLK phosphorylation.

Combining previous data, the following scenario is proposed (Fig. 25). Newly synthesized CLK accumulates in the cytoplasm in a hypo-phosphorylated state. CYC binding stabilizes and transfers CLK to the nucleus (§ V.1.a), where medium-phosphorylated CLK forms activate transcription of downstream target genes. Hyper-phosphorylation as well as degradation of CLK are subsequent to its export to the nucleus and take place in the cytoplasm. Importantly, according to this hypothesis soluble (not linked to chromatin) hyper-phosphorylated CLK co-exists with hypo-phosphorylated newly synthesized CLK at the beginning of the day in fly extracts.

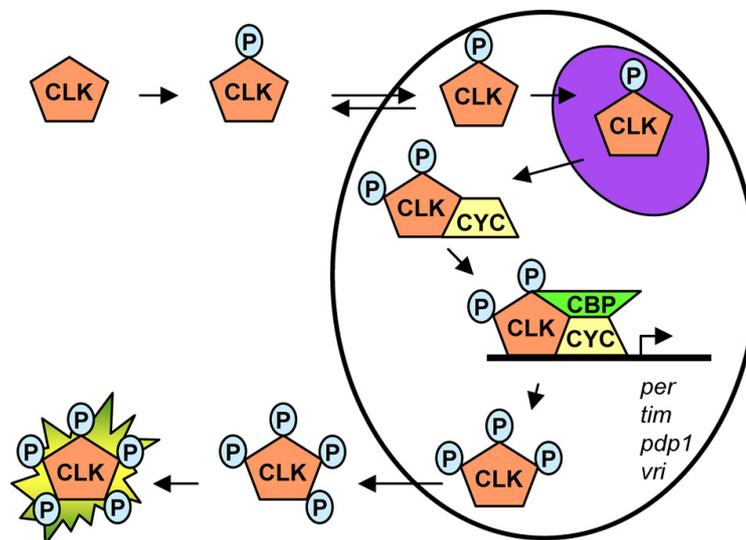


Figure 25 A progressive phosphorylation regulates localization of the CLK protein.

Once synthesized, CLK is first phosphorylated in the cytoplasm. Low-phosphorylated CLK enter the nucleus and possibly accumulate in nuclear foci. Further phosphorylated CLK recruits CYC and activate transcription of clock genes (*per*, *tim*, *vri*, *pdp1*). Additional phosphorylation is accompanied by CLK release from chromatin (in a PER/DBT-containing complex) and transcriptional inhibition. Hyper-phosphorylated CLK exits the nucleus. In the cytoplasm, extra phosphorylation triggers CLK degradation (Hung et al., 2009)

f. CLK quantities are crucial for circadian and cellular functions

Timed *Clk* gene transcription and post-translational modifications coordinate the availability of constant CLK protein amounts during a daily cycle. CLK-mediated transcription is sensitive to high CLK levels. Multiplying the number of *Clk* cDNA copies in flies induces a progressive shortening in the locomotor period length accompanied by higher transcriptional rates (Kadener et al., 2008). Likely, more CLK induces an enhanced transcription of *per* which allows the PER repression on CLK target genes transcription to come sooner and the period of the cycle to get shorter. Overexpression of a UAS-*Clk* transgene using a *tim*-GAL4 driver causes lethality, while overexpression in a more restricted pattern of cells (*cry*-GAL4 or *clk*-GAL4) do not, meaning that lethality comes from the broad exogenous pattern of expression of the *tim*-GAL4 driver.

The *Clk^{Jrk}* mutation, a loss-of-function mutation with dominant negative properties, has no consequences on viability. However, homozygous *Clk^{Jrk}* mutant flies show no rhythmic locomotor activity under free-running conditions; heterozygous flies are half arrhythmic and those rhythmic show slightly longer period than wild-type controls (Allada et al., 1998).

CLK expression is thought to be the minimum requirement necessary to generate circadian cycles in a non-circadian cell (Zhao et al., 2003). In addition, CLK is essential for proper development and functioning of clock neurons, since from larval stages. In *Clk^{Jrk}* larvae, s-LN_v neurons show aberrant projections and a loss of PDF expression. Homozygous *Clk^{Jrk}* adults show a marked overgrowth of l-LN_v neurons with enhanced aberrant projections, while s-LN_{v,s} are absent. Similar results are found in *cyc⁰* mutants, but neuronal defects are less prominent (Michard-Vahnee et al, in preparation). In addition, overexpression of a more stable *Clk* mRNA (bearing a modified SV40 3'UTR region) in *pdf*-positive cells forces the appearance of novel *pdf*-positive neurons around LN_{v,s} (S. Kadener - personal communication).

In conclusion, CLK quantities has to be restricted within a limited range to assure adequate both developmental and cellular functions.

2. Regulation of CLK/CYC-mediated transcription

Transcription of *Drosophila* E-box-containing “clock” genes is driven by the CLK/CYC heterodimeric transcription factor. *cyc*⁰ mutants lose rhythmic mRNA expression patterns, revealing a role for CYC in circadian gene transcription (Rutila et al., 1998). However, cultured *Drosophila* S2 cells – that possess endogenous CYC but no or undetectable quantities of CLK, show inefficient expression of clock genes, like *per* or *tim*. Transfection of a transcriptionally potentiated version of CYC (CYC-VP16, bearing the VP-16 viral transcription activator domain) was still unable to drive expression from E-boxes-containing promoters (Kadener et al., 2008). CLK transfection activates basal transcription from E-boxes (*per*, *tim* expression as well as luciferase signal from *tim*-luc reporter transgenes), indicating that CLK is necessary for circadian transcription.

The activity of the CLK/CYC heterodimer likely can be assigned to the C-terminal polyglutamine region on CLK: this region is almost entirely lost in *Clk*^{irk} mutants, which show low transcriptional rates.

a. The repressor complex

PER and TIM nuclear entry during night is accompanied by decrease in the levels of *per* and *tim* transcripts, indicating repression of CLK/CYC transcriptional activity (Bae et al., 2000). Similarly, PER transfection in S2 cells inhibits transcription from *tim*-luc reporters (Darlington et al., 2000). Co-immunoprecipitation assays in fly head extracts confirm a temporally regulated CLK/PER/DBT and CLK/TIM complexes formation that coincides with transcriptional repression (Kim et al., 2007; Sun et al., 2010; Menet et al., 2010). CYC protein is present in large excess compared to CLK quantities, nevertheless, PER or PER/TIM preferentially interact with CLK in the PER/CLK/CYC complex (Bae et al., 2000).

in vitro and *in vivo* studies have shown the capacity of PER alone (independently from TIM) to inhibit CLK/CYC transactivation of E-box-regulated genes: *per* or *tim* genes, or *per-luc* reporter gene expression (Rothenfluh et al., 2000; Ashmore et al., 2003; Chang and Reppert, 2003). However, recent evidences indicate that PER does not directly inhibit CLK/CYC-mediated transcription but likely function as a scaffold for the interaction of uncharacterized repressor partners with the CLK/CYC heterodimer (Kim et al., 2007; Menet et al., 2010; Sun et al., 2010). In *per*⁰, *per*^Δ flies – that express a *per* transgene deficient for

the DBT-binding domain on PER, the PER Δ protein is still able to bind both CLK and TIM but behaves as a very poor repressor (Kim et al., 2007). On the other hand, in *per*^{ACBD} flies – expressing a *per* transgene coding for a mutant PER protein unable to bind CLK, the PER^{ACBD} protein is still able to account for repression of CLK/CYC transcription in flies (Sun et al., 2010). Therefore, transcriptional repression requires a PER-containing complex but not PER direct binding to CLK. TIM is present in a complex with CLK/CYC during the night when peak of transcriptional repression occurs (Menet et al., 2010) and TIM somehow strengthens PER repressive activity on CLK. Indeed, TIM acts as a bridge to link together the PER (PER^{ACBD}) and CLK proteins (Sun et al., 2010).

In addition to TIM, other protein partners were proposed to control CLK/CYC-mediated gene transcription. *clockwork orange (cwo)* is a clock-controlled gene (its expression cycles over a day). Based on *in vitro* studies, the encoded protein, CWO, was supposed to synergize with PER to block transcription of the CLK/CYC target genes: *per*, *tim*, *vri*, *Pdpl* and *cwo* itself (Kadener et al., 2007). However, levels of the corresponding protein at the peak of their oscillations were higher in wild-type flies than in *cwo* mutants (Richier et al., 2008), suggesting that *in vivo* CWO acts as an activator – instead of a repressor, for transcription of clock genes.

kayak (kay), the *Drosophila* homolog of *c-fos*, encodes a bZip transcription factor that was shown to negatively regulate transactivation of CLK target genes (Ling et al., 2012), likely through promoting the expression of a CLK/CYC repressor. In addition, KAY controls *Clk* gene transcription through VRI (Fig. 24, § V.1.b).

b. The CLK/CYC transcription factor cyclically binds to chromatin

Drosophila CLK/CYC binding to DNA for clock genes expression is timely regulated. CLK/CYC binding to E-boxes only occurs at times when target genes are being actively transcribed. Indeed, anti-CLK chromatin immunoprecipitation (ChIP) assays demonstrates a cyclical binding signal of CLK on *per* and *tim* promoters (Menet et al., 2010), that starts during late day and peaks around ZT14 (Abruzzi et al., 2011) before going back to low levels few hours later, at times when PER binding is also seen. Likely, the PER/TIM/DBT-containing repressor complex inhibits transcription by removing CLK from E-boxes (Yu et al., 2006; Menet et al., 2010).

Circadian transcription of CLK target genes comprehends these subsequent iterative phases (Abruzzi et al., 2011) (Fig. 26):

1. CLK binding to E-boxes during the day, with a peak during evening-time (ZT14).
2. Few hours later (peak at ZT18), CLK-bound to chromatin is recognized by the PER protein or a PER-containing complex that starts inhibition of target gene transcription. Anti-PER ChIP can be found at this time, but not less stringent PER-CLK IP complexes, suggesting that CLK and PER may be still bound to chromatin. This defines an “on-DNA” PER-CLK inhibition phase.
3. This phase is followed by an “off-DNA” PER-CLK complex (starting from ZT19), where CLK and PER co-immunoprecipitates in a likely 1:1 stoichiometric ratio in non-chromatin-bound dimers. This is the time when a strong nuclear TIM staining is seen.
4. The day after, newly synthesized or probably re-activated (through dephosphorylation) CLK binds again to chromatin for a new cycle.

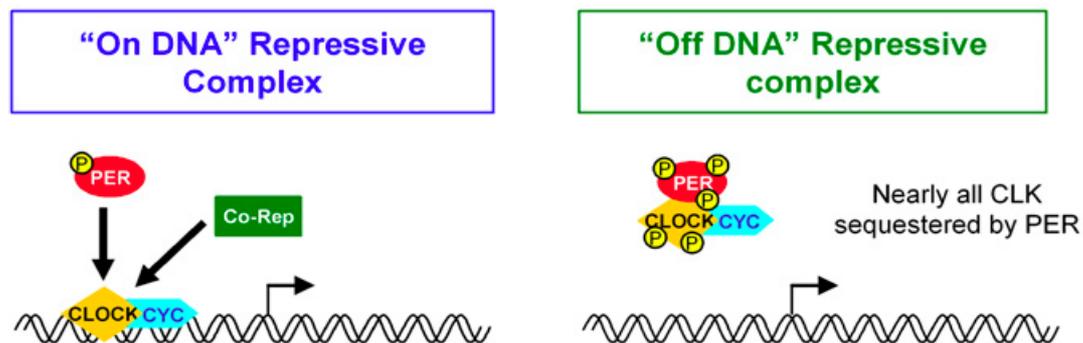


Figure 26 Model for the repression of transcription of CLK/CYC target genes.

At midnight, PER binds to clock genes promoters and starts the inhibition of transcription in an “on-DNA” repressive complex. Few hours later, PER has sequestered CLK in an “off-DNA” repressive complex (Menet et al., 2010).

However, a basal above-background CLK ChIP signal on E-boxes of clock-regulated genes is visible all-day long (Abruzzi et al., 2011), suggesting that chromatin is never completely inaccessible and other co-activators (or repressor) could account for stronger (or weaker) cyclical DNA binding at specific time-points.

c. Post-translational modifications regulate CLK transcriptional ability

Western blot analysis of the CLK protein in head extracts shows bands in a variable migrating range, the extent of which oscillates during a 24h cycle. Hyper-phosphorylated CLK accumulates during the late night-early morning when transcription of CLK target genes

is low, and hypo-phosphorylated, fast-migrating CLK bands are the only present at peaks of transcriptional activation. Therefore, CLK phosphorylation state appears to be strongly related to its transcriptional ability.

Indeed, mutants for clock proteins that eventually affect CLK phosphorylation, like *dbt* (*dbt^P*, *dbt^{ar}*, overexpression of *dbt^{K/R}*) or *per* (*per Δ* or *per^{ACBD}*), manifest altered CLK target gene transcription. Moreover, mutants that induce stabilization of hyper-phosphorylated CLK show very low *per*, *tim* or *vri* transcription rates (Kim et al., 2007; Yu et al., 2009; Sun et al., 2010). Dephosphorylation events may contribute to replenish hypo-phosphorylated, transcriptionally active, CLK amounts. PP2A and PP1 phosphatases function within the oscillator (§ IV.4.d), and downregulation of *tws* and *wdb* PP2A regulatory subunits was shown to destabilize CLK in cultured cell assays (Kim and Edery, 2006).

Importantly, ubiquitylation was also shown to be a determining feature for CLK/CYC transcriptional activity. CLK undergoes daily ubiquitylation cycles and maximum ubiquitylation coincides with peaks of CLK/CYC target gene transcription (Luo et al., 2012). Deregulation of USP8, a de-ubiquitylating enzyme, by expression of a dominant-negative form or by RNAi, induces higher quantities of *per*, *tim* and *vri* transcripts but has only a mild effect on CLK protein stability. USP8 interacts with CLK in cultured cells and affects its ubiquitylation both in S2 cells and *in vivo*. Together, the data suggest that USP8-mediated CLK deubiquitylation down-regulates CLK/CYC transcriptional ability and transcriptionally active CLK is thus ubiquitylated (Luo et al., 2012). On the other hand, knockdown of the E3 ubiquitin ligase CTRIP also alters transcription of CLK-target genes, possibly through PER (Lamaze et al., 2011).

Remarkably, circadian transcription factors were shown to function in fungi (*N. crassa*) and mammals as “kamikaze” activators: highly unstable FRQ and CLOCK/BMAL1 are the most active, while proteasome inhibition results in long-lived but less potent activators (Schafmeier et al., 2008; Stratmann et al., 2012). These considerations may also be applied to *Drosophila*. Mono-ubiquitylated and maximally active CLK at the end of the day may follow two alternative fates: it can be further ubiquitylated (by CTRIP) and targeted for proteasome degradation, which promotes rapid inactive CLK recycling; or deubiquitylated (by USP8), which minimizes its transcriptional ability at late night-early morning (Luo et al., 2012).

VI. THE PP2A PHOSPHATASE

Protein phosphatase 2A (PP2A) is the most important serine/threonine phosphatase family in metazoa. Extremely well conserved among distant species (Table 2), it accounts for up to 1% of total cellular proteins in some tissues or organs, like the brain (Janssens and Goris, 2001; Slupe et al., 2011). PP2A has a pivotal role in a huge number of cellular processes. PP2A functions are critical for cell physiology, including cell cycle regulation, cell proliferation and death, development, cytoskeleton dynamics and regulation of multiple signal transduction pathways; it is also an important tumor suppressor protein (Janssens and Goris, 2001; Shi, 2009).

1. PP2A: a mainly tripartite holoenzyme composed of A/B/C subunits

The core PP2A enzyme is a dimer of a catalytic subunit (PP2A_C) and a scaffold subunit (PR65 or PP2A_A). A third regulatory subunit, named B subunit, can associate to this core structure (Fig. 27 – Table 2) (Janssens and Goris, 2001). Four different families of regulatory subunits have been identified, termed B, B', B'', B''' families. The association of these third subunits to the core dimer is mutually exclusive, meaning only one single B-type subunit can bind the core dimer each time.

This multiple combinatorial association is central to the mechanisms that regulate PP2A activity and ensure the pleiotropic role of this important enzyme (Martin et al., 2010). Formation of the heterotrimer is essential for PP2A stability, since knockdown of anyone class of subunits (A, C, or all B subunits) reduces both the abundance and the stability of all the other subunits (Gallego and Virshup, 2005). In addition, a number of interacting cofactors can bind the dimer and/or the holoenzyme.

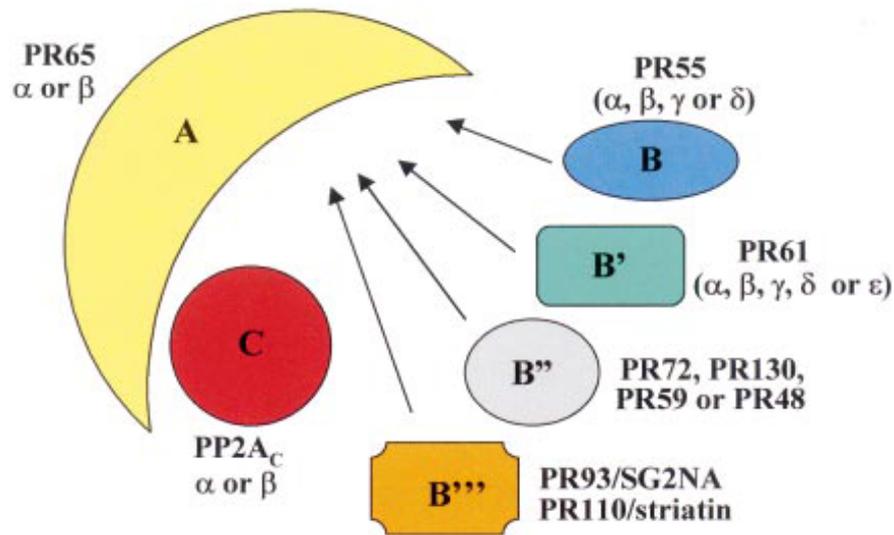


Figure 27 The PP2A holoenzyme composition.

PP2A consists predominantly of a heterotrimer, with conserved A (scaffold) and C (catalytic) subunits and variable B-type regulatory subunit. There are four B subunit gene families, each with two to five genes and many multiple splice variants. (Janssens and Goris, 2001)

a. The PP2A A/C core dimer

The PP2A core dimer, consisting of the catalytic (C) and the scaffold (A) subunits, represents at least one-third of total cellular PP2A protein (Janssens and Goris, 2001).

Mammals possess two distinct catalytic subunits - encoded by two different genes, PP2A_Cα and PP2A_Cβ. In *Drosophila*, only one PP2A_C isoform exists, which was named *microtubule star (mts)* accordingly to the phenotypes shown by *mts* mutants (elongated arrays of microtubule filaments irradiating from centrosomes, which reminds of a star). Sequence and structure comparison between very distant species, from yeast to fungi, plants, insects and humans, reveals that the structure of PP2Ac has remained remarkably constant throughout evolution, making PP2A one of the most conserved of all known enzymes (Janssens and Goris, 2001). Expression of PP2Ac within cells is tightly controlled by auto-regulatory transcriptional and post-translational mechanisms that ensure constant levels of PP2A activity. Free PP2A_C (not in complex with regulatory subunits) is unstable, and even ectopic expression cannot substantially increase PP2A_C amounts (Sents et al., 2013). Moreover, PP2A_C is synthesized in an inactive form, preventing non-specific PP2A activity that would

be extremely risky for cellular functions. PP2A_C activation is tightly coupled to holoenzyme assembly and involves a number of PP2A binding partners (§VI.2).

Tightly associated to the C subunit is the A subunit, which forms a structural scaffold for the recruitment of B-type subunits, as well as additional proteins. Different B subunits interact via the same or overlapping sites within the A subunits, which explains why binding of the B subunit is mutually exclusive. The structure of the A subunit consists of a 15 tandem-repeated motifs, known as HEAT (huntingtin-elongation-A subunit of PP2A-TOR), each one formed by two superimposed α -helices. Repeats are stuck together to arrange an elongated C-shaped architecture with a continuous hydrophobic core. The scaffold subunit exhibits high conformational flexibility that accommodates binding of the catalytic subunit and other interacting proteins, such as regulatory subunits (Shi, 2009). As for the catalytic subunit, mammals express two distinct A/PR65 isoforms, while the *Drosophila* homolog is unique (PP2A-29B).

	<i>H. sapiens</i>	<i>D. melanogaster</i>	<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>N. crassa</i>
PP2A C	PP2A _C α PP2A _C β	Mts	Pph21 Pph22	Ppa3p	Pp2A
PP2A A/PR65	PR65 α PR65 β	Pp2A-29B	Tpd3	Paa1p	
PP2A B/PR55	PR55 α PR55 β PR55 γ PR55 δ	Tws	Cdc55		Rgb-1
PP2A B'/B56/PR61	PR61 α PR61 β PR61 γ PR61 δ PR61 ϵ	Wdb Wrd	Rts1		
PP2A B''/PR72	α /PR72/PR130 β /PR59 γ /PR48	CG4733			
PP2A B'''/PR93/PR110	PR110/Striatin PR93/SG2NA mMOB1	Cka	Far8	Csc3p	Ham-3

Table 2 Homologs of PP2A subunits in mammals (*H. sapiens*), fruit fly (*D. melanogaster*), yeast (*S. cerevisiae*, *S. pombe*) and fungi (*N. crassa*).

b. The regulatory B-type families of subunits

The striking feature of regulatory subunits is their diversity. Four different families of B subunits have been distinguished – B/PR55, B'/PR61, B''/PR72, B'''/PR93/PR110, with extremely low sequence similarities among them. Despite the lack of sequence similarity, B-type subunits do recognize similar overlapping motifs on the A subunit. Each family comprises different genes, and isoforms arise from alternative splicing. The spatial and temporal expression patterns of regulatory subunits within tissues, cells or subcellular locations can be highly divergent.

B subunits are characterized by the presence of multiple organized motifs and surfaces that mediate various protein-protein interactions. In particular, B subunits also connect the holoenzyme to compartment-specific adaptors (such as cytoskeleton proteins) and help to place PP2A in vicinity to its targets.

In *Drosophila*, PP2A functions regulate a large number of cellular and developmental processes. The fruit fly B-type subunit TWINS (TWS) works as a positive regulator for the Wingless (WG) signaling cascade (Bajpai et al., 2004; Zhang et al., 2009). In addition, TWS functions are required for proper imaginal disc organization and neural cell identity (Uemura et al., 1993). A *Drosophila* WIDERBORST (WDB) B'-type regulatory subunit-containing PP2A enzyme acts within the Hedgehog signaling to regulate Smoothed (Smo) and Cubitus interruptus (Ci) dephosphorylation and localization (Jia et al., 2009). In addition, WDB plays a role in planar cell polarization by localizing the PP2A to the distal pole of microtubules, at the level of apical junctions (Hannus et al., 2002). TWS and WDB activities also impact the fly circadian behaviors (Sathyanarayanan et al., 2004, § IV.4.d). Another B'-type *Drosophila* regulatory subunit, WELL-ROUNDED (WRD), mediates PP2A functions at the motoneuron synaptic bouton by regulating the microtubule structure at the neuromuscular junction (Viquez et al., 2009). WRD also de-phosphorylates the S6K kinase, one of the most important effectors of the insulin/TOR signaling cascade (Hahn et al., 2010). The *Drosophila* CG4733 gene codes for a B''-type PP2A regulatory subunit whose biological role has not been investigated yet. CONNECTOR OF KINASE TO AP-1 (CKA) has recently been found as the fruit fly homolog of vertebrate Striatins, which compose the large majority of the B'''-type regulatory subunits group in vertebrates (Ribeiro et al., 2010). CKA controls *Drosophila* organ size and tissue growth through the Hippo signaling pathway.

2. Regulation of PP2A

The functions of the PP2A holoenzyme are regulated at multiple levels. Primarily, the association of a defined B-type subunit mediates localization of the PP2A holoenzyme to specific cell compartments. Moreover, methylation at the C-terminus of the catalytic subunit polypeptide regulates B-subunit binding. In addition, the core enzyme associates with other interacting partners that cooperate to modulating PP2A stability and functions (Fig. 28).

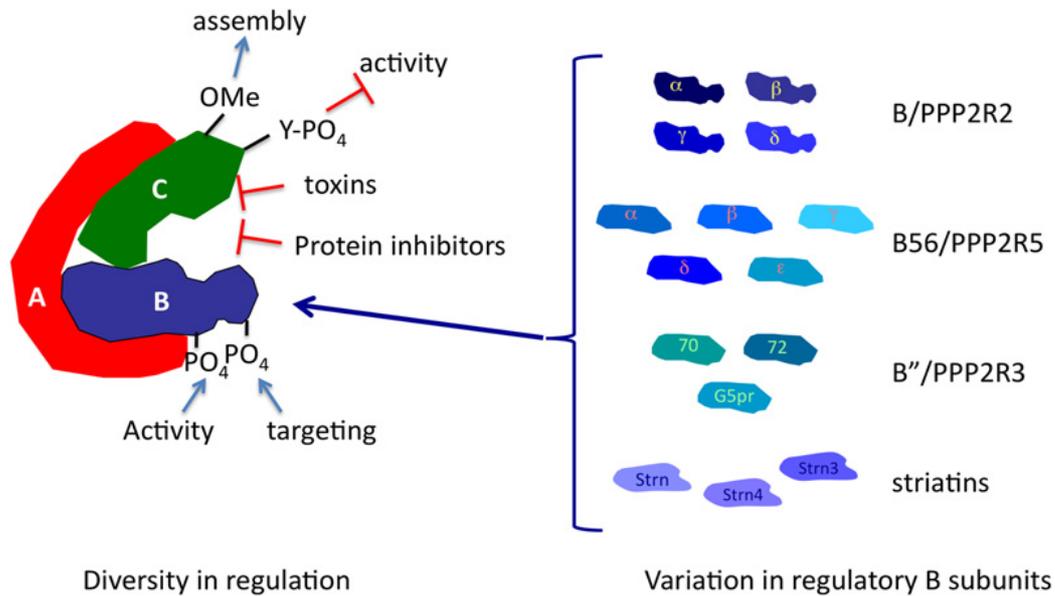


Figure 28 Regulation of the PP2A holoenzyme (vertebrates).

The PP2A holoenzyme is regulated at multiple levels: toxins (as Okadaic acid), protein inhibitors and phosphorylation of the B and C subunits regulate activity, holoenzyme assembly and substrate specificity (Virshup and Shenolikar, 2009)

a. Holoenzyme composition

Thanks to the broad diversification of regulatory subunits in cellular localization and tissue-specific expression, B subunits association to the core dimer dictates the substrate specificity of the holoenzyme and targets PP2A activities to specific cell compartments. Moreover, B subunits mediate interactions with compartment-specific partners, notably the cytoskeleton, thus helping in localizing the PP2A in vicinity to its targeted substrates and place of action. Various mechanisms of post-translational modification can affect specific B-type subunit association and B-subunit exchange into the holoenzyme.

b. Post-translational modifications: phosphorylation and methylation

Activity of the PP2A enzyme is also regulated by post-translational modifications.

Phosphorylation of the catalytic subunit on specific Thr and Tyr residues (Thr304 and Tyr307) at the C-terminus inactivates PP2A. Nevertheless, PP2A can rapidly re-activate itself in an auto-dephosphorylation reaction, which implicates that PP2A can also act as a phosphotyrosine phosphatase (PTPase). These modifications interfere with holoenzyme assembly by selectively inhibiting interaction with certain regulatory subunits (Sents et al., 2013). Regulatory subunits (in particular B'-type) are also subjected to phosphorylation events that modulate holoenzyme formation and/or activity.

In addition, PP2A is sensitive to methylation at the conserved most C-terminal Leu residue (Leu309) on the catalytic subunit (Janssens and Goris, 2001, Shi, 2009, Sents et al., 2013) (Fig. 29). While its implications on phosphatase activity are controversial, C subunit methylation regulate PP2A complex formations through enhancing the affinity of the core dimer for some (B), but not all (B'), regulatory subunits (Martin et al., 2010; Shi, 2009). By regulating holoenzyme assembly, methylation influences PP2A_C cellular location (Shi, 2009).

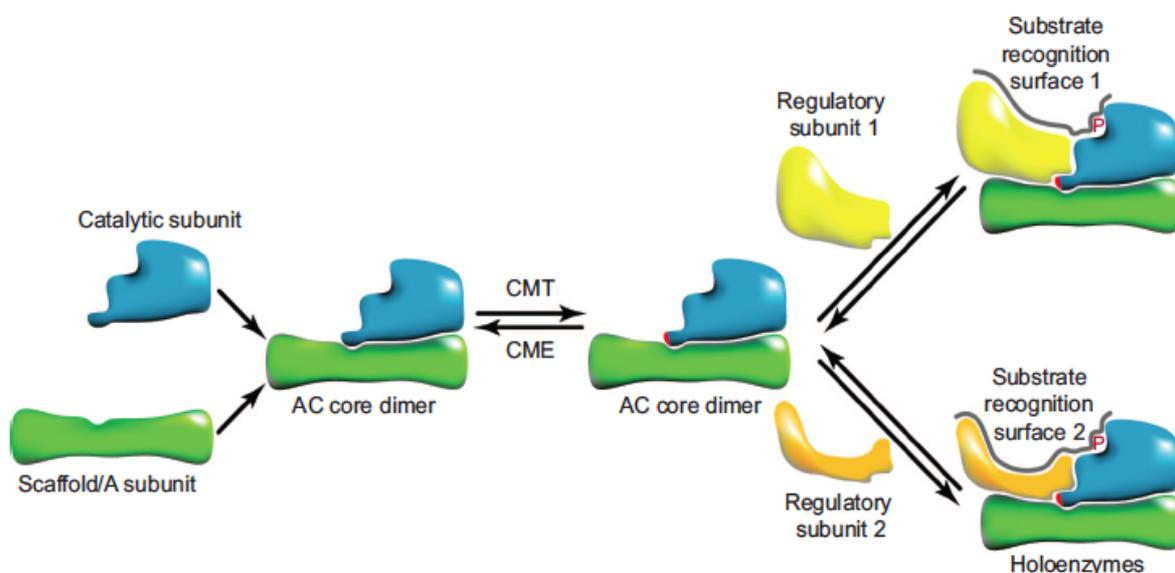


Figure 29 The catalytic subunit of PP2A undergoes reversible methylation.

Methylation is catalyzed by a C-terminal leucine methyltransferase (CMT) and demethylation is catalyzed by a PP2A-specific carboxymethyl esterase (CME). Methylation facilitates the binding of (some) regulatory subunits and formation of holoenzymes with distinctive, peculiar substrate-recognition surfaces. (Mumby, 2007)

c. Other PP2A binding partners: PTPA and $\alpha 4$

A number of interacting proteins have been isolated that differentially regulate the PP2A core enzyme.

PP2A tyrosine phosphatase activator (PTPA, even named *phosphatase two A phosphatase activator*) was originally identified as an ATP/Mg²⁺-dependent enzymatic up-regulator of phosphotyrosyl phosphatase activity of PP2A *in vitro*, but there is to day no *in vivo* clear evidence for that. Yet, PTPA was shown to promote activation of the Ser/Thr phosphatase activity of a natively inactive PP2A form, thus confirming a role for PTPA in regulating PP2A activity and biogenesis. For this, PTPA is thought to interplay with PME-1, the PP2A methyl-esterase enzyme. In fact, PME-1 directly binds to the active site of *de novo* synthesized PP2A_C and stabilizes the catalytic subunit in an inactive form (Virshup and Shenolikar, 2009; Sents et al., 2013). PME-1 plays thus an additional role towards PP2A, which is independent to its esterase functions: it stabilizes PP2A_C.

$\alpha 4$, also known as Tap42 or IGBP1, forms a stable complex directly with PP2A_C. Indeed, $\alpha 4$ competes with the A subunit for PP2A_C binding on separate but partially overlapping residues on the catalytic subunit (Herzog et al., 2012). Thanks to its structurally disordered C-terminal tail, $\alpha 4$ can directly link phospho-substrates to the PP2A catalytic subunit for dephosphorylation. $\alpha 4$ can also bind the catalytic subunits of other PP2A-related phosphatases (PP4 and PP6), and depletion of $\alpha 4$ correlates with a general loss of phosphatase activities that causes cell lethality. $\alpha 4$ also participates to PP2A stabilization, notably by protecting PP2A_C from proteasome-mediated degradation until functional phosphatase complex are assembled (Herzog et al., 2012).

d. PP2A inhibitors

TIPRL1 is a ubiquitously expressed inhibitory protein that binds the catalytic subunits of PP2A (as well as that of PP2A-like enzymes: PP4, PP6) and with so prevents promiscuous phosphatase activity. Moreover, SET and CIP2A are two endogenous molecules that directly contact the catalytic site and displace one or the two catalytic metal ions required for phosphate hydrolyzation, leading to inactivation of the PP2A catalytic subunit.

Okadaic acid (OA) is a potent tumor-inducing toxin produced by dinoflagellate algae that specifically inhibits PP2A by occupying the surface pocket on the active site of the PP2A catalytic subunit. OA also inhibits PP1 but with less affinity compared to PP2A. Calyculin A

(CalA) is also commonly used as a PP2A inhibitor drug, but its inhibitory activity is largely more specific to Ser/Thr phosphatases than PP2A in particular.

3. Mechanisms of PP2A substrate recognition

Studies focused on direct binding, like yeast-two-hybrid assays or co-immunoprecipitations, have highlighted the fact that PP2A do physically interact with the target substrate it dephosphorylates (Virshup and Shenolikar, 2009).

Substrate recognition by PP2A involves additive effects of multiple discrete interactions with both the catalytic and the regulatory subunits. Hence, well-defined linear sequences or common consensus motifs within PP2A substrates cannot be identified. PP2A substrate specificity arises from (Slupe et al., 2011):

1. *Tissue and subcellular localization of PP2A* defined by the B subunit. Regulatory subunit genes (and gene isoforms) are differently expressed in tissues and regulatory proteins localize to different cellular and subcellular compartments, giving rise to different holoenzyme complexes in separate cell locations. PP2A localization is also given by interaction between the PP2A holoenzyme complex and other effectors.

2. *Selective holoenzyme assembly* by post-translational modifications. Incorporation of specific regulatory subunits into the holoenzyme is influenced by reversible phosphorylation and methylation of the C-terminal tail of the PP2A catalytic subunit. Moreover, phosphorylation of the B subunits may affect holoenzyme assembly and activity.

3. *Interaction with specific endogenous inhibitors*

4. *Substrate recruitment by regulatory subunit interactions distant to and near the active site.* B subunits engage weak electrostatic interactions away from the catalytic cleft of the C subunit, which increases local substrate concentration. Then, B subunits interaction near the active site orientates the phosphopeptides to mediate site-specific dephosphorylation.

4. PP2A super-complexes

Protein phosphatases reverse kinase activity by dephosphorylating the substrates of those kinases. Remarkably, one of the major classes of phosphatase substrates is the kinases themselves, whose dephosphorylation is generally accompanied with their inactivation. On

the other hand, as previously discussed, phosphatase activity is also (negatively) regulated by kinase-mediated phosphorylation (Janssens and Goris, 2001). PP2A was shown to modulate the activities of several kinases, like the ERK/MAPKs, calmodulin-dependent kinases and PKA, both *in vitro* and *in vivo*. To this regard, more and more studies have given evidences for the existence of “super-complexes” where both kinases and phosphatases coexist and reciprocally act as substrates for the enzymatic activity of the counteracting partner. In these “signaling modules” kinases and phosphatases regulate their own activities within a self-correcting signaling complex.

Given the relevance of complex composition in the biological functions of PP2A, various studies have recently investigated the interacting partners of PP2A-containing molecular assemblies in humans (Goudreault et al., 2009; Glatter et al., 2009; Herzog et al., 2012). All researches recapitulate the presence of several trimeric PP2A complexes, each containing an exclusive regulatory B-type subunit, as well as non-conventional PP2A complexes. In particular, studies reported the existence of a highly connected PP2A sub-network surrounding the canonical PP2A C/A/Striatin (B’’) complex. Protein network comparisons with homologs in other species revealed that this mapped interaction module has been highly conserved within evolution.

5. The STRIPAK complex

By iterative affinity purification/mass spectrometry (AP/MS) approaches in cultured cells, Goudreault et al. (2009) and Glatter and coworkers (2009) identified a novel human PP2A super-complex surrounding the PP2A A/C dimer - also found by Herzog and coworkers (2012) by chemical cross-linking/mass spectrometry (XL/MS) assay. This sub-network contains members of the striatin B’’ class of PP2A regulatory proteins, the Striatin-interacting protein Mob3, members of the Germinal Center Kinase-III (GCK-III) class of serine/threonine kinases (MST4, STK24, STK25), the protein CCM3, as well a specific group of structurally related proteins (STRIP1, STRIP2) (Fig. 30). Authors named this novel multiprotein assembly *striatin-interacting phosphatase and kinase* (STRIPAK) complex. More recently, Hoydo and colleagues (Hoydo et al., 2012) defined MISSHEPEN-LIKE KINASE 1 (MINK1), another member of the Ste-20 related kinases, as an additional component of the STRIPAK complex.

a. Main STRIPAK components

Striatins (Strn) are conserved intracellular proteins highly enriched in brains, especially at the synapses, where they are thought to participate to endocytosis and vesicular trafficking (Gordon et al., 2011). The striatin structure contains, from the N- to C- terminus, a putative caveolin-binding motif, a coiled-coil domain, a Ca²⁺-calmodulin binding motif and a large WD-repeat domain, all of which are known to mediate protein-protein interactions. For this high concentration in binding domains, striatins are thought to serve as adaptors for protein complexes and/or as a scaffold for dynamic signaling cascades. In addition, the disordered coiled-coil domain mediates homo-dimerization. Deletion experiments showed that striatin N-terminal portion is essential for mediating interaction with PP2A and Strip proteins, whereas the C-terminal region is sufficient for interaction with Mob3 and GckIII kinases (Kean et al., 2011). Hence, striatin functions in the human STRIPAK complex as a scaffold to bridge the GCKIII family of kinases and the PP2A phosphatase (Gordon et al., 2011; Hyodo et al., 2012).

Germinal center kinases, members of the sterile-20 (Ste-20) kinase group, are phosphoproteins implicated in cell migration, cell cycle progression, apoptosis and stress response pathways (Dan et al., 2001). They were shown to localize to the Golgi apparatus (Kean et al., 2011), where they participate in Golgi assembly and proper cell orientation. Auto-phosphorylation at the catalytic site is believed to increase activity of Ste-20 kinases (Gordon et al., 2011).

Mob3 is a striatin-interacting protein whose functions have not yet been fully established. Yet, Mob3 is involved in vesicular trafficking (Gordon et al., 2011) and participates to kinase activation in fungi (Bloemendal et al., 2012) and yeast (Frost et al., 2012). In addition, Mob3 interacts with the light chain of the dynein motor complex, suggesting a role in cytoplasmic trafficking (Goudreault et al., 2009).

Strip-1 and 2 (also known as Fam40-A and B) are high-related proteins of largely unknown function in metazoa. Sequence analysis shows the presence of three putative transmembrane domains and a conserved central Pro-rich region. Moreover, Strip proteins exhibit a highly conserved C-terminal domain showing up to 27% identity to homologous sequences from other organisms (Kemp and Sprague, 2003; Goudreault et al., 2009; Bloemendal et al., 2012). Strip proteins functions have been associated to actin cytoskeleton remodeling, cell shape and cell migration (Bai et al., 2011).

b. The STRIPAK complex in Drosophila and other organisms

Orthologs of mammalian STRIPAK components have been reported to exist in many eukaryotes (Table 3). This underlies their importance in key biological processes.

A paper in 2010 (Ribeiro et al., 2010) described the existence of a *Drosophila* PP2A-containing complex highly related to human STRIPAK (Fig. 30). Around MTS (PP2A_C) and Pp2A-29B (PP2A_A), the *Drosophila* STRIPAK complex contains Connector of kinase to AP-1 (CKA), which is reported as the only fruit fly homolog of human Striatins, and MOB4, homolog of human Mob3. CKA and MOB4 interact with CG11526, the *Drosophila* homolog of human Strip; two Ste-20-like kinases of the germinal center kinase family, Misshapen (MSN) and Germinal center kinase III (GCKIII) are also bound to CKA and MOB4.

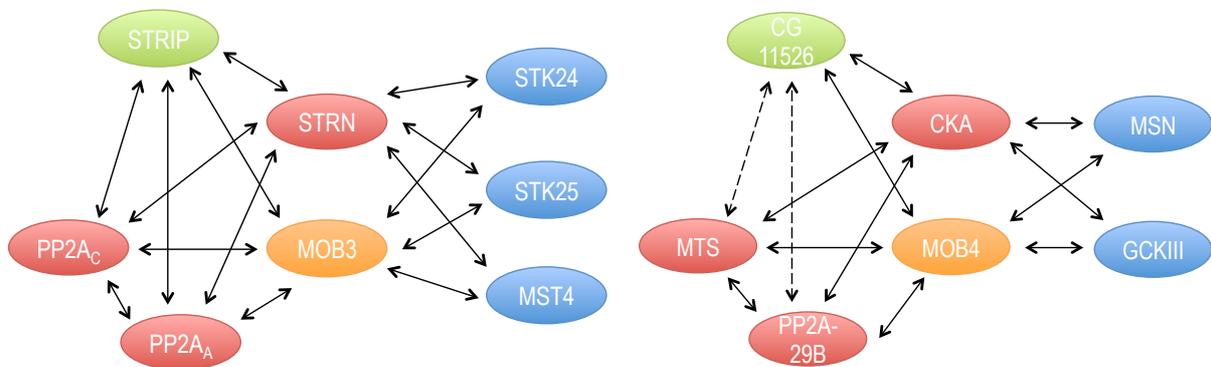


Figure 30 The major components of the STRIPAK complex in humans (left) and flies (right).

Around PP2A A/C/B''' subunits (red) is the B'''-interacting Mob protein (orange), which forms a core STRIPAK network. The Strip protein (green) associates to this main group of proteins, which also independently interacts with members of the Ste-20 kinase family (blue). Rearranged from (Goudreault et al., 2009; Ribeiro et al., 2010).

Components of the STRIPAK complex are conserved from yeast to humans. Ancestral roles for STRIPAK point to cytoskeletal and membrane dynamics functions.

In *N. crassa* orthologs of STRIPAK components are required for hyphal fusion and sexual propagation. In the ascomycete *S. macrospora* gene products for various STRIPAK components (PRO11/Striatin, PRO22/Strip, SmMOB3/Mob3, SmPP2AA/PP2A_A) play a central role in regulating sexual development (fruiting body formation) and vegetative hyphal fusion (Bloemendal et al., 2012). In this study authors propose that the transmembrane region of PRO22 (Strip), anchors the scaffolding protein PRO11 (Striatin) - and other STRIPAK components, to the cytoplasm. In the fission yeast *S. pombe* six homolog components of the

mammalian STRIPAK complex have been isolated, including Striatin and Strip homologs (Frost et al., 2012) that together form a correlation group. STRIPAK proteins localize to the mitotic spindle pole body during early mitosis and likely interact with components of the actomyosin contractile ring to regulate the formation of the septation initiation network (SIN) that mediates proper cytokinesis and mitotic transitions.

In *S. cerevisiae*, Far8 (Striatin), Far11 (Strip), Far10 as well as Far3 and Far7 are known since longtime to form a large protein complex (Far complex) implicated cell cycle arrest following pheromone treatment (Kemp and Sprague, 2003; Pracheil et al., 2012). Proteins of the Far complex participates to interaction with a number of nuclear and/or RNA-regulatory proteins, first suggesting that the Far complex may localize to the nuclear envelope or the endoplasmic reticulum membrane contiguous with the nuclear envelope. A recent paper (Pracheil and Liu, 2013) showed that the Far/STRIPAK complex components are progressively recruited to the endoplasmic reticulum (ER): Far3/7/8 proteins are recruited to the ER by Far9 and Far10 - which are integral ER membrane proteins; the complex in turn recruits Far11 to the ER. Localization of Far complex at the ER is necessary for the PP2A/Far complex functions in the TORC2 signaling.

SPECIE		<i>H. sapiens</i> (human)	<i>D. melanogaster</i> (fruit fly)	<i>S. cerevisiae</i> (budding yeast)	<i>S. pombe</i> (fissing yeast)	<i>N. crassa</i> (fungus)	<i>S. macrospora</i> (fungus)
STRIPAK/FAR COMPLEX COMPONENTS	PP2A catalytic	PP2A _c α PP2A _c β	Mts	Pph21 Pph22	Ppa3p Ppa1/Ppa2	PP2Ac	SmPP2Ac
	PP2A scaffold	PP2A _s α PP2A _s β	PP2A-29B	Tpd3	Paa1	PP2AA	SmPP2AA
	PP2A regulatory B'''	Strn Strn3 Strn4	Cka	Far8	Csc3p	HAM-3	PRO11
	MOB	Mob3/Phocein (MOBK3)	Mob4	Mob1	Mob1p	MOB-3	SmMOB3
	STRIP	STRIP1/2 (FAM40A/B)	CG11526	Far11	Csc2p	HAM-2	PRO22
	FGFR1 oncogene partner	SIKE/FGFR1OP2	CG10158/FGOP2	Far3 Far7	Csc4p		
	Sarcolemma associated protein	SLMAP	CG17494	Far9 Far10	Csc1p	HAM-4	PRO45
	Cortactin-binding protein	CTTNBP2	CG10915				
	Cerebral cavernous malformation 3	CCM3 (PDCD10)	CG5073				
	GCKIII proteins (Ste-20 related kinases)	MST4	CG5169/GckIII				
MST1/2 STK24		Hpo					
STK25/MINK1		Msn					

Table 3 Components of the STRIPAK complex in different species. Only components that are known to form a complex or perform similar cellular functions are listed (Bloemendal et al., 2012; Pracheil and Liu, 2013).

c. Biological functions of the STRIPAK complex

The human STRIPAK complex consists of a kinase-phosphatase network module in which the PP2A phosphatase directly controls dephosphorylation and inactivation of members of the Ste-20 germinal center kinases, MST3 and MINK1 (Kean et al., 2011; Gordon et al., 2011; Hyodo et al., 2012). Moreover, *Drosophila* and mammalian STRIPAK complexes were shown to bind to and negatively regulate the Hippo kinase pathway (Ribeiro et al., 2010; Hata et al., 2013).

MST3 is involved in Golgi orientation and assembly ((Kean et al., 2011), while MINK1 kinase is required during the septation processes that allow proper cell cytokinesis (Hyodo et al., 2012). Furthermore, in *S. pombe*, the STRIPAK-like complex negatively regulates the septation initiation network (SIN) during mitosis processes (Frost et al., 2012). In *S. cerevisiae*, PP2A-Far11 complex antagonizes TORC2 signaling. In the TOR (target of rapamycin) signaling, TOR complex 2 (TORC2) has a separate specific function compared to TORC1, which involves cell-cycle dependent polarization of the actin cytoskeleton (Pracheil et al., 2012; Pracheil and Liu, 2013). Eventually, a number of STRIPAK components including Striatins and Strip1/2 associates with dynein, a motor protein that localizes to the mitotic spindle and controls the transport of protein trafficking (Hutchins et al., 2010). All of these studies reveal an evolutionary conserved implication of the STRIPAK complex in mitosis, cytoskeleton organization and membrane fusion dynamics.

Both in mammals (Hata et al., 2013) and in *Drosophila* (Ribeiro et al., 2010), the phosphatase activity of STRIPAK complex has been shown to negatively affect the Hippo (Hpo) kinase cascade, which is the major regulator of growth control and organ size in a variety of organisms. Together with the yeast STRIPAK complex implication in the TOR signaling (Pracheil et al., 2012), this relation underlies a possible role of the STRIPAK complex in regulating developmental processes and apoptosis. The Hippo pathway is also a newly emerging signaling that functions as a tumor suppressor, suggesting that anti-tumoral properties of PP2A may also be achieved through the STRIPAK complex signaling.

STRIPAK components in human and in *S. cerevisiae* localize to the ER/nuclear envelope (Frost et al., 2012; Pracheil and Liu, 2013). The Golgi-bound Strip interacts with SLMAP bound to the nuclear envelope, thus bridging the Golgi and the outer nuclear membrane together with the centrosome. These interactions likely occur during mitosis and assure proper cytokinesis, suggesting a functional role for the STRIPAK complex throughout the cell cycle (Frost et al., 2012).

Interestingly, aggravating interaction between *S. pombe* Far complex genes and another B-type PP2A subunit, B'/Par1, was shown, indicating that alternative B subunits may compensate for the Far/Striatin-mediated PP2A specificity (Frost et al., 2012). Likewise, a Far11-PP2A/Rts-1 complex, with Rts-1 being a B'-type PP2A regulatory subunit, have been proposed in *S. cerevisiae* to antagonize TORC2 activity (Pracheil et al., 2012). These data suggest that alternative B-type subunit-containing PP2A complexes may take part or associate to the STRIPAK network.

VII. OBJECTIVE OF THE THESIS

The pace of the circadian clock largely depends on post-transcriptional and especially post-translational modifications of clock proteins, which primarily create a delay between repressor gene(s) expression and repressor protein(s) accumulation. Phosphorylation governs the stability, subcellular localization and activity of PER and TIM proteins as well as stability and DNA-binding properties of the CLK protein. A number of kinases, phosphatases and ubiquitin-ligases have been found that modify core clock components, but more are required to understand how the *Drosophila* circadian oscillator generates a 24h pace.

A major problem in the search for new clock genes is that most of these enzymes are also involved in other cellular pathway and may be required during development, making adult mutants hard to isolate. The Gal4-UAS system is a powerful technique that allows targeting of transgene expression only in a restricted subset of cells, and can be used to direct the expression of RNAi transgenes only to clock neurons. RNA interference (RNAi) is an evolutionary conserved process of endogenous post-transcriptional gene silencing that has been largely exploited by researchers to drive knockdown of proteins of interest.

In the laboratory, we took advantage of an extended collection of RNAi transgenic flies (R. Ueda, NIG-FLY collection, Japan) to set up a large-scale behavioral screen using the Gal4-UAS system to target gene silencing selectively to LN_v neurons – that are known to control rhythmic locomotor behaviors under constant dark conditions (DD). “Positive” RNAi-expressing flies were selected based on altered locomotor activity rhythms in DD (longer or shorter periods, or arrhythmicity). Downregulated genes that correspond to the strongest behavioral defects were selected for further analysis. My PhD project aimed at deciphering the functions of some of these genes within the *Drosophila* circadian molecular clockwork.

RESULTS and DISCUSSION

RESUME DE LA THESE

Analyse de nouveaux gènes impliqués dans le contrôle des rythmes veille-sommeil chez *Drosophila melanogaster*

En raison de la rotation de la terre autour de son axe, tous les êtres vivants sont soumis à des variations quotidiennes de lumière, de température et d'humidité. Au cours de l'évolution, les organismes ont développé une horloge interne leur permettant d'anticiper ces variations environnementales et d'organiser leur physiologie et leurs comportements afin qu'ils correspondent à la période la plus favorable de la journée.

Cette horloge interne prend le nom de circadienne, du latin *circa* = environ et *diem* = un jour. Par définition, elle fonctionne indépendamment de la présence d'un stimulus externe récurrent, c'est à dire qu'elle persiste même dans des conditions environnementales constantes d'obscurité et de température. Toutefois, elle utilise les stimuli environnementaux, notamment la lumière, pour se synchroniser sur les cycles jour-nuit. Les stimuli extérieurs, appelés *Zeitgebers* (de l'allemand « donneur de temps »), modifient la phase des rythmes circadiens. Enfin, la période du rythme (24h) reste constante quelle que soit la température extérieure (les rythmes circadiens sont compensés en température).

Depuis les années 70, avec la découverte des premiers mutants pour le gène d'horloge *period (per)* (Konopka and Benzer, 1971), la drosophile représente un puissant système modèle pour la chronobiologie. Les mouches présentent des rythmes comportementaux contrôlés par l'horloge circadienne et facilement analysables, comme le rythme d'activité locomotrice. De plus, les outils génétiques développés chez *Drosophila melanogaster* en ont fait un modèle phare pour l'étude des mécanismes moléculaires et cellulaires sous-tendant la génération des rythmes circadiens.

L'activité locomotrice de la mouche est contrôlée par un groupe de 150 « neurones d'horloge » situés dans le cerveau central. De plus, des horloges autonomes sont aussi présentes dans les organes et tissus périphériques. Cela nous permet d'étudier les oscillations des protéines d'horloge sur différents tissus, tels que les extraits de têtes, où la majeure contribution est donnée par l'horloge des yeux.

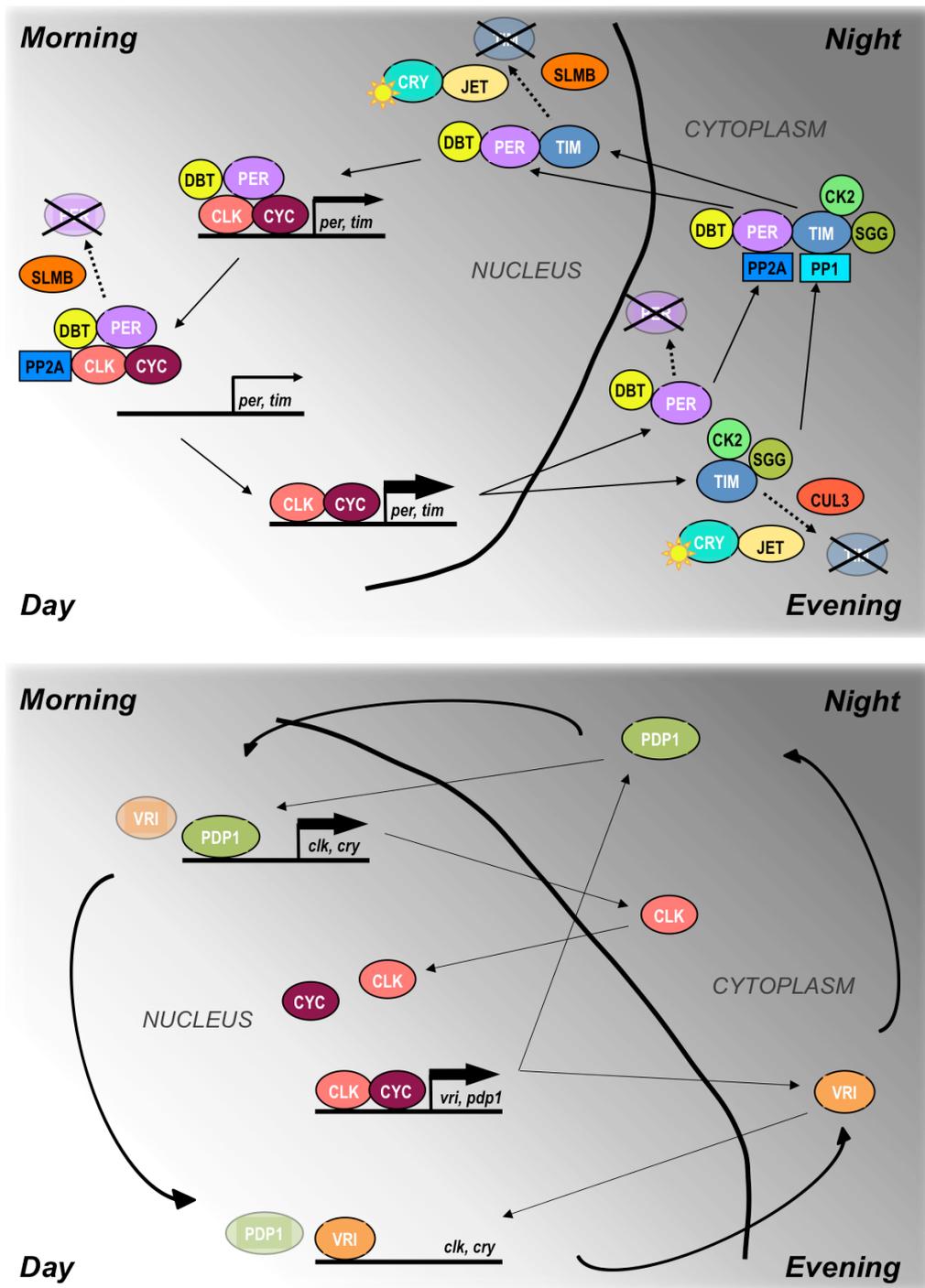


Figure 1 L'horloge moléculaire chez *Drosophila melanogaster*

En haut Schéma présentant la boucle de rétroaction transcriptionnelle de *per/tim*. Le dimère CLOCK/CYCLE (CLK/CYC) active l'expression des gènes *period* (*per*) et *timeless* (*tim*). Par la suite, les protéines PER et TIM s'accumulent dans le cytoplasme, avant d'être transloquées dans le noyau, où elles inhibent l'activité transcriptionnelle de CLK/CYC, ainsi que leur propre expression. Couplée à cette boucle, une deuxième boucle de rétroaction transcriptionnelle de *Clk* existe (**en bas**), au cours de laquelle CLK/CYC active la transcription des gènes *vri* (*vri*) et *pdp1*, deux facteurs de transcription qui sont, respectivement, l'inhibiteur et l'activateur de l'expression du gène *Clk*.

Les mécanismes moléculaires contrôlant les rythmes circadiens sont conservés parmi les organismes des différents règnes (plantes, animaux et champignons). Ils se composent de boucles de rétroaction où un complexe d'activation transcriptionnelle entraîne l'expression des répresseurs de son activité.

Chez la drosophile, l'horloge moléculaire est composée de deux boucles de rétrocontrôle négatif, centrées sur un couple de facteurs de transcription (Hardin, 2011) (Fig.1). Dans une première boucle, le couple de facteurs de transcription CLOCK (CLK)/CYCLE (CYC) active la transcription des gènes *period* (*per*) and *timeless* (*tim*) à la fin de la journée. Les protéines PER et TIM s'accumulent dans le cytoplasme de la cellule et forment des complexes au cours de la nuit. Par la suite, elles sont transloquées dans le noyau, où, en fin de nuit, elles s'associent avec le complexe CLK/CYC pour en inhiber la fonction transcriptionnelle, et donc l'activation des gènes *per* et *tim* eux-mêmes. Le matin, les protéines PER et TIM sont dégradées par le système ubiquitine-protéasome, ce qui permet à CLK et CYC de démarrer un nouveau cycle de transcription.

Dans une seconde boucle, le complexe CLK/CYC active la transcription des gènes *vri* (*vri*) et *PAR domain protein 1ε* (*pdp1ε*), deux facteurs de transcription régulant *Clk*. La protéine VRI, en venant se fixer sur son promoteur, inhibe l'expression du gène *Clk* à la fin de la journée. Ensuite PDP1ε, en entrant en compétition avec VRI, active la transcription de *Clk* en fin de nuit (Cyran et al., 2003).

Ces boucles d'autorégulation génèrent des oscillations des ARNm des gènes cibles de CLK/CYC (Fig.2). Toutefois, celles-ci ne sont rendues possibles que si les étapes d'activation et de répression transcriptionnelles sont temporellement décalées. De nombreux mécanismes post-transcriptionnels et post-traductionnels, principalement basés sur des étapes de phosphorylation, permettent de réguler l'accumulation et l'activité à la fois des composants positifs et négatifs de la boucle. Ces modifications causent des oscillations des protéines CLK, PER et TIM en quantité et en poids moléculaire, tout au long d'un cycle de 24h, clairement visibles par Western blot. De nombreuses kinases (Doubletime (DBT)/CK1, CK2, Shaggy (SGG)/GSKIII), phosphatases (Protéine Phosphatase 1 (PP1) et PP2A), et ubiquitine ligases (Supernumerary Limbs (SLMB), Cullin-3 (CUL-3) and Jetlag (JET)) modifient les protéines PER, TIM et CLK afin de contrôler leur localisation, leur activité et surtout leur stabilité.

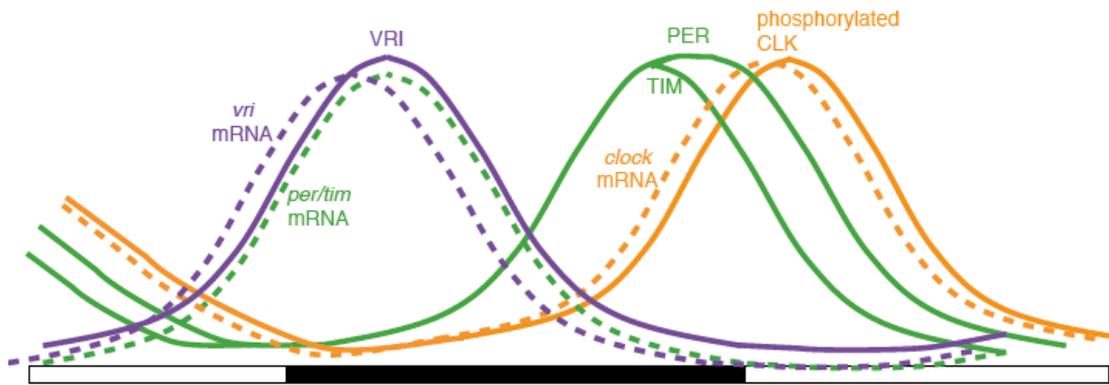


Figure 2 Oscillations des ARNm et protéines d'horloge.

L'expression des gènes *per*, *tim* et *vri* est décalée de 12h par rapport au cycle d'ARNm du gène *Clk*. Les protéines VRI et CLK s'accumulent en phase avec leur ARNm ; au contraire, il existe un délai d'accumulation entre les ARNs et les protéines PER et TIM.

Les mécanismes de contrôle post-traductionnel des protéines PER et TIM ont été largement étudié (Harms et al., 2004; Tataroglu and Schafmeier, 2010). En revanche, peu de données sont actuellement disponibles sur les mécanismes régulant la stabilité et l'activité du facteur de transcription CLK. Comme pour PER et TIM, CLK suit un cycle de phosphorylation de 24h, se caractérisant par des formes hypo-phosphorylées au début de la nuit et passant progressivement à des formes de haut poids moléculaire (hyper-phosphorylées) en fin de cycle, le matin (Yu et al., 2006). Il existe une corrélation entre le niveau de phosphorylation de CLK et l'activité transcriptionnelle du dimère CLK/CYC. En effet, la transcription des gènes *per* et *tim*, ainsi que celle d'autres cibles, est maximale au début de la nuit, quand CLK est peu phosphorylé, et minimale le matin, quand CLK est fortement phosphorylé. Ainsi, la phosphorylation de CLK est corrélée à son inactivation (Yu et al., 2006; Menet et al., 2010). En plus de la formation du CLK nouvellement synthétisé, des mécanismes de déphosphorylation ont été proposés pour la formation d'une forme hypo-phosphorylée de CLK transcriptionnellement active (Hardin, 2011). Des études en culture de cellules ont montré que la phosphatase PP2A, avec les sous-unités régulatrices Widerborst (WDB) et Twins (TWS), contrôlait la stabilité du CLK transfecté (Kim and Edery, 2006).

Chez la drosophile comme dans l'ensemble des Métazoaires, la PP2A est un complexe enzymatique tripartite, composé d'une sous-unité catalytique (C), d'une sous-unité scaffold (A), et d'une sous-unité régulatrice (B) (Janssens and Goris, 2001). Quatre différentes familles de sous-unités régulatrices, chacune composée d'un ou plusieurs membres, sont

connues. Chaque protéine régulatrice reconnaît une même région sur le complexe catalytique-scaffold, ce qui rend la liaison entre la sous-unité catalytique et la sous-unité régulatrice mutuellement exclusive. En effet, la sous-unité régulatrice joue un rôle clé : elle détermine la spécificité du substrat à déphosphoryler en prenant contact avec la protéine phosphorylée sur plusieurs sites (à cause de cela, un motif de séquence spécifique pour la reconnaissance du substrat ne peut pas être isolé) (Virshup and Shenolikar, 2009). De plus, la sous-unité régulatrice prend aussi contact avec des protéines du cytosquelette, ce qui favorise une localisation subcellulaire spécifique de la PP2A.

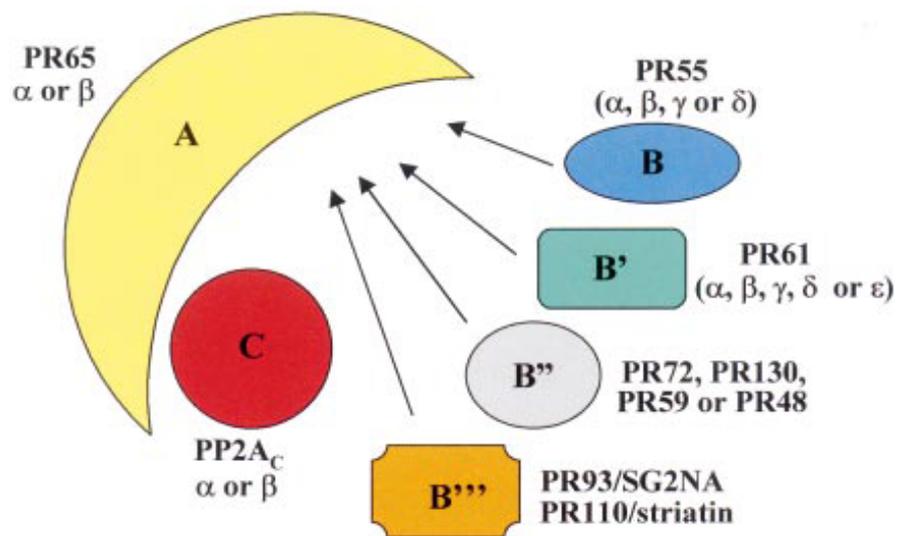


Figure 2 Composition de l'enzyme PP2A

L'enzyme PP2A est composée de 3 différentes sous-unités. La sous-unité catalytique (C) s'associe à une sous-unité scaffold (A) et forme un premier dimère « cœur ». Une troisième sous-unité, appelée régulatrice (B), s'associe à ce dimère. Quatre différentes familles de sous-unités régulatrices ont été identifiées (B, B', B'', B'''); chacune reconnaît une même région de liaison sur le dimère cœur, ce qui fait que la liaison au dimère A/C est mutuellement exclusive entre les différentes sous-unités de type B (Janssens and Goris, 2001).

Chez la drosophile, une seule sous-unité catalytique existe et elle prend le nom de *microtubule star*, MTS. La sous-unité scaffold dans la mouche est appelée PP2A-29B. Parmi les sous-unités régulatrices connues on retrouve Tws (groupe B), Wdb et Well-rounded -Wrd (groupe B'), CG4733 (groupe B'') et Connector of Kinase to AP-1 (Cka), récemment identifié comme appartenant au groupe B''' (homologue des Striatins chez l'homme) (Ribeiro et al., 2010).

L'activité des phosphatases peut être contrôlée par phosphorylation (Janssens and Goris, 2001), et une des classes majeures de substrats pour la PP2A sont les kinases elles-mêmes. De

plus, des super-complexes contenant à la fois des kinases et des phosphatases ont été repérés. Initialement découvert dans une étude sur les cellules humaines (Goudreault et al., 2009), le complexe STRIPAK (Striatin-interacting phosphatase and kinase) se compose de la sous-unité catalytique et scaffold de la PP2A, une Striatin (PP2A regulatory subunits du type B'''), la protéine Strip (transmembranaire) et Mob, qui se lie au cytosquelette, et des membres de la famille Germinal center du groupe des kinases Ste-20. Des orthologues des protéines composant le complexe STRIPAK de mammifères ont été retrouvés dans d'autres eucaryotes : les champignons (*N. crassa* et *S. macrospora*) (Bloemendal et al., 2012), la levure (*S. cerevisiae* et *S. pombe*) (Kemp and Sprague, 2003; Pracheil and Liu, 2013), les insectes (*D. melanogaster*) (Ribeiro et al., 2010). Le complexe STRIPAK est impliqué dans les réarrangements du cytosquelette contrôlant les processus de mitose et/ou fusion cellulaire (Pracheil and Liu, 2013; Frost et al., 2012). Par son rôle dans les mécanismes d'apoptose, le complexe STRIPAK joue aussi un rôle dans le développement et la croissance des cellules et organes (Pracheil et al., 2012; Ribeiro et al., 2010). Enfin, par liaison avec Strip, qui se localise à proximité du Golgi et du réticulum endoplasmique, le complexe STRIPAK se positionne à proximité de l'enveloppe nucléaire, où il peut contrôler le transfert des protéines du et vers le noyau (Pracheil and Liu, 2013) (Frost et al., 2012).

Comme dit précédemment, les mécanismes de modification post-traductionnels sont essentiels pour la détermination de la période du cycle. La phosphorylation contrôle la stabilité, la localisation subcellulaire et l'activité répressive des protéines PER et TIM, ainsi que la stabilité et l'activité transcriptionnelle de la protéine CLK. Un certain nombre de partenaires qui modifient les principaux composants d'horloge (kinases, phosphatases et ubiquitine ligases) ont été isolés, mais d'autres sont attendus pour le réglage fin de la boucle.

Afin d'isoler des nouveaux composants de l'horloge moléculaire chez la drosophile, nous avons mis en place une étude comportementale basée sur l'expression ciblée de transgènes ARNi (ARN interférence) dirigés contre la moitié du génome de *Drosophila* (Fig. 3). Grâce au couplage avec le système GAL4-UAS, les transgènes ARNi ont été exprimés spécifiquement dans les neurones d'horloge de la mouche connus pour être responsables de la rythmicité en conditions constantes (s-LNvs, driver *gal1118-GAL4*).

Cinquante-quatre gènes potentiels d'horloge ont été identifiés. Parmi eux, certains ont été signalés pour fonctionner au sein de l'horloge moléculaire de la mouche par d'autres groupes de recherche, ce qui prouve la validité de notre crible.

Mon projet de thèse a eu comme but l'étude du rôle de certains de ces gènes potentiels d'horloge, sélectionnés pour le fort défaut comportemental induit par l'expression du ARN correspondant.

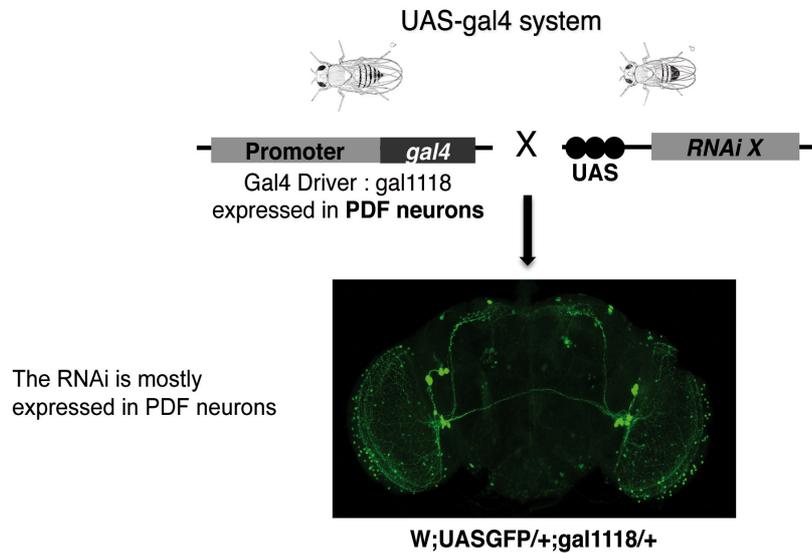


Figure 3 Bases génétiques du crible ARNi

Le driver *gal1118*-GAL4 induit une forte expression du reporter UAS-*gfp* dans les neurones s-LNVs (exprimant le neuropeptide PDF), qui assurent la rythmicité de la mouche en conditions constantes. Les mouches *gal1118*-GAL4 ont été croisées par les mouches exprimant une séquence ARNi spécifique (base de lignées transgéniques de NIG-FLY, Mishima, Japan), et leur descendance est testée pour leur rythme d'activité locomotrice en conditions d'obscurité constante pendant 7 jours. Les génotypes qui montrent un altération de la période (période plus courte ou plus longue que 24h, ou absence de rythmicité) ont été re-testées.

Dans un premier temps, les effets de le ARNi sur le gène cible ont été validés à la fois en comportement, par l'utilisation d'autres séquences ARNi dirigées contre des portions différentes de la séquence du gène cible, et également par l'observation d'une baisse de quantité de l'ARNm du gène cible. Par ailleurs nous avons complété l'étude grâce à d'autres approches génétiques (mutants, insertions d'éléments transposables).

CG17350 est l'un des gènes retenu dans le crible. L'expression du transgène *CG17350*-RNAi élimine les oscillations de la protéine PERIOD dans les neurones d'horloge de la mouche adulte. Cependant, les oscillations de PER dans les neurones de l'horloge larvaire des mutants nuls pour *CG17350* ne sont pas affectées (données non montrées). Les effets de l'expression du transgène RNAi sont donc vraisemblablement causés par la dérégulation d'un gène autre que *CG17350* et non encore identifié.

Le gène *CG12082* de la drosophile est l'orthologue de l'Ubiquitin-specific protéase 5 (USP5) chez l'homme. La dérégulation d'USP5 par ARNi au sein des cellules d'horloge allonge la période de l'activité locomotrice des mouches ainsi que les oscillations de la protéine PER dans les neurones d'horloge. L'étude des extraits de têtes de mouches montre une accumulation des formes de haut poids moléculaire des protéines PER et TIM chez les ARNi *usp5* pendant la journée, alors que ces protéines sont normalement dégradées dans un contexte sauvage. Ces résultats suggèrent un défaut de dégradation de ces protéines dans les extraits ARNi (Fig.4 en haut). De plus, TIM n'est pas stabilisé en absence de PER (mutant *per⁰*), alors que PER est toujours stabilisé par l'expression du ARNi *usp5* même en absence de TIM (*tim⁰*) (Fig.4, en bas). En conséquence, USP5 participe directement à la dégradation de la protéine PERIOD (PER) et, dans un deuxième temps, de la protéine TIMELESS. En outre, USP5 contrôle également la stabilité de la protéine CLOCK.

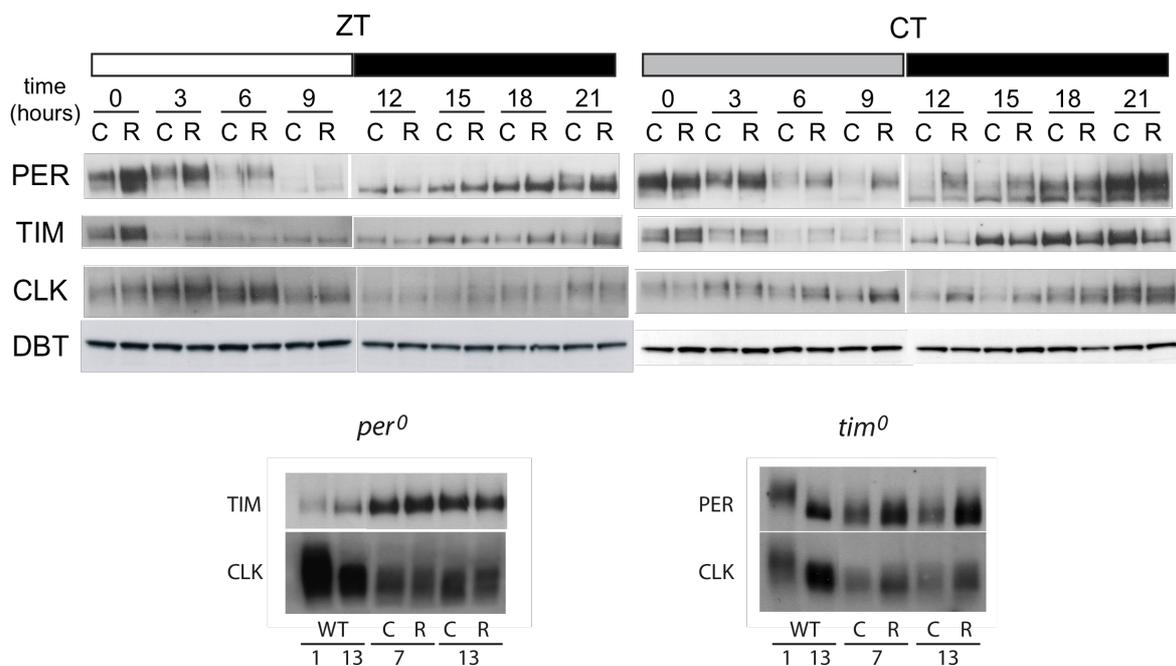


Figure 4 Stabilisation de formes à haut poids moléculaire après expression du ARNi contre *usp5*

Eh haut : Analyse par Western blot des extraits de têtes de mouches sauvages (contrôle, C) et exprimant le ARNi *usp5* (RNAI, R) collectées toutes les 3 heures dans des conditions « normales de 12 heures de lumière-12 heures d'obscurité (à gauche) et en condition d'obscurité constante (à droite). Profil des oscillations moléculaires des protéines PER, TIM et CLK. Un marquage DBT est utilisé comme contrôle de chargement sur gel. Un rectangle blanc, gris ou noir montre les périodes de lumière réelle ou subjective, ou d'obscurité, respectivement. ZT = temps avec *zeitgeber* (lumière), CT= temps circadien (pas de signal synchronisateur). **En bas:** Analyse par Western blot des extraits de têtes de mouches dans des conditions génétiques mutantes pour *per* (à gauche, *per⁰*) ou *tim* (à droite, *tim⁰*). Les temps de récolte des mouches sont indiqués en bas. WT = sauvage (*per⁺*, *tim⁺*).

Etant une ubiquitine protéase, USP5 peut directement déubiquitinyler PER. Cependant, d'après le rôle décrit chez l'homme, USP5 joue un rôle spécifique dans le désassemblage des chaînes de poly-ubiquitine présentes en forme libre dans la cellule. Des études récentes démontrent que ces polymères d'ubiquitine peuvent activer des voies de signalisation cellulaire, ainsi que jouer sur l'activation de certaines kinases. De manière plus importante, ces chaînes peuvent également entrer en compétition active avec les protéines ubiquitinylées pour la reconnaissance au niveau du protéasome, bloquant leur dégradation. Donc, USP5 peut contrôler la dégradation de la protéine PER par son activité de désassemblage des chaînes libres d'ubiquitine. Des expériences complémentaires sont toutefois nécessaires afin de mieux décrire le mécanisme selon lequel USP5 régule la dégradation des protéines d'horloge, dont PER.

La plus grande partie de mon travail de thèse a été effectué sur le gène *CG11526*, dont les fonctions étaient encore inconnues. En raison de la forte homologie avec la protéine humaine Strip chez l'homme, nous avons décidé de l'appeler Strip.

Strip interagit avec la protéine CONNECTOR OF KINASE TO AP-1 (CKA), sous-unité régulatrice de la phosphatase PP2A, à la fois dans les cellules S2 d'insectes et dans les extraits de têtes de mouches. La dérégulation à la fois de Strip et/ou de CKA amène à des phénotypes comportementaux de période longue, se caractérisant par un retard dans les cycles d'oscillations de la protéine PERIOD au sein des neurones d'horloge. De plus, des formes de hyper-phosphorylées de la protéine CLOCK s'accumulent dans la matinée quand CKA et/ou Strip sont perturbées (Fig. 5). La perturbation des activités globales de la PP2A (surexpression d'une forme dominant négative de la sous-unité catalytique, *mts^{DN}*) produit également une hyper-phosphorylation de CLK la matin, suggérant que les complexes PP2A/CKA/STRIP sont impliqués dans le contrôle de la déphosphorylation de CLK à la fin du cycle (données non montrées).

PP2A a été montré pour affecter la stabilité de CLK dans les cellules S2 d'insectes. En effet, la dérégulation soit de WISERBORST (WDB) ou TWINS (TWS), deux sous-unités régulatrices différentes de la PP2A, induit une forte baisse de la protéine CLK dans les cellules transfectées (Kim and Edery, 2006). Nous avons montré que la diminution de WDB affecte également la stabilité du CLK dans les extraits de têtes de mouches, mais n'a aucun effet apparent sur les niveaux de phosphorylation de la protéine CLK (Fig.6). De plus, l'expression de le ARNi contre *wdb* diminue également les quantités des protéines PER et

TIM, sans affecter leur cycle de phosphorylation (Fig. 6). La déstabilisation de CLK, PER et TIM est toutefois accompagnée par un très faible allongement de la période de l'activité locomotrice des mouches (25h).

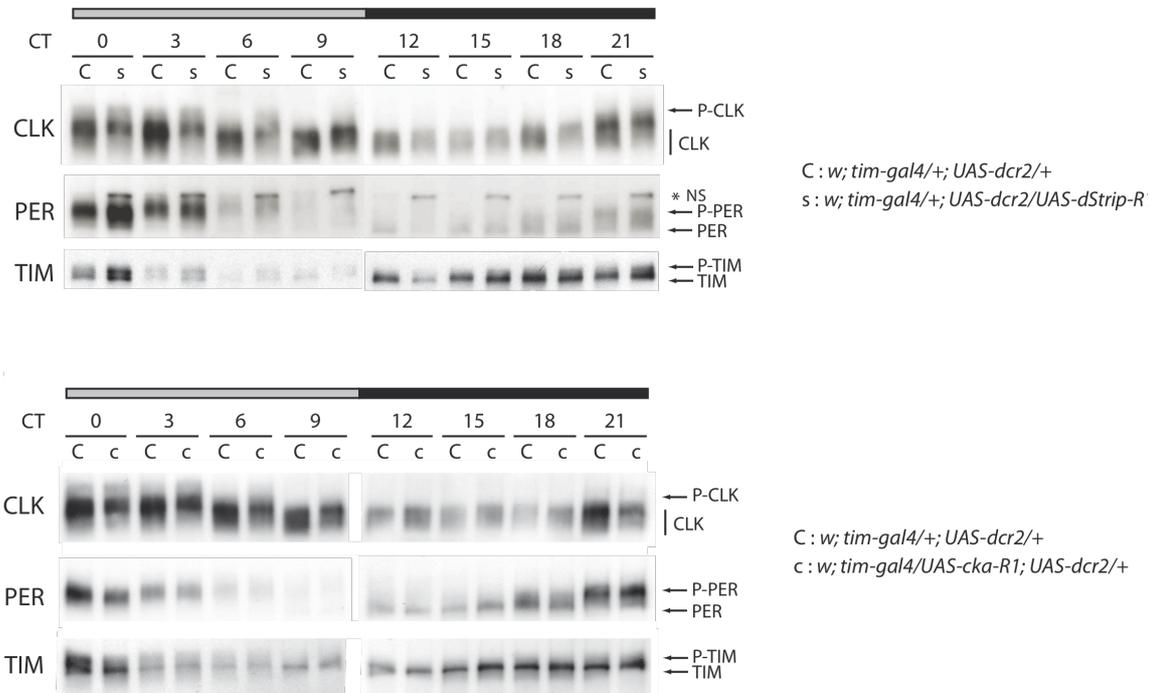


Figure 5 Hyper-phosphorylation de la protéine CLK pendant la journée subjective dans les mouches dérégulées par *Strip* et *Cka*

Analyse par Western blot des profils moléculaires de PER, TIM et CLK sur des extraits de têtes de mouches récoltées toutes les 3 heures pendant le premier jour d'obscurité constante (temps circadien, CT). En haut, les effets de la dérégulation de la protéine *Strip*, en bas, les effets de l'expression du ARNi *Cka*.

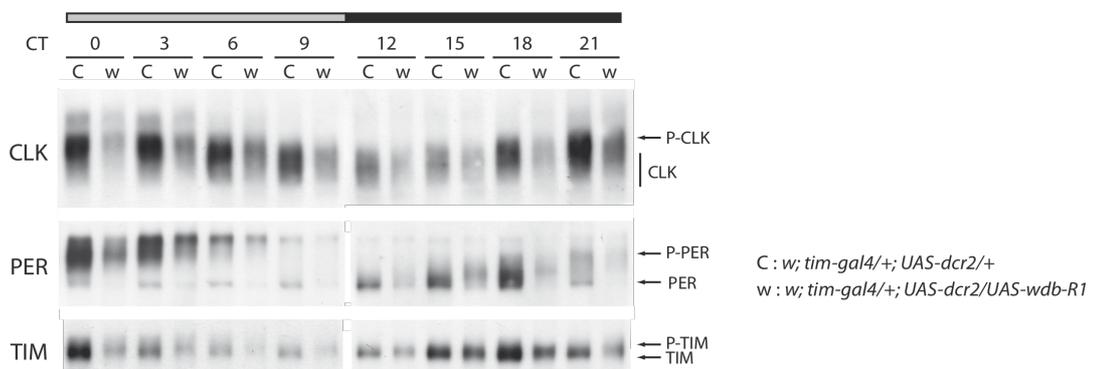


Figure 6 Déstabilisation des protéines d'horloge dans les mouches dérégulées par *wdb*.

Analyse par Western blot des profils moléculaires de PER, TIM et CLK sur des extraits de têtes de mouches sauvages et exprimant le ARNi contre *wdb*, récoltées toutes les 3 heures pendant le premier jour d'obscurité constante (temps circadien, CT).

Il existe ainsi une corrélation négative entre le niveau de phosphorylation de CLK et sa capacité transcriptionnelle : le CLK hyper-phosphorylé du matin est inactif alors que le CLK hypo-phosphorylé, présent au début de la nuit, serait le plus actif transcriptionnellement. En accord avec les effets d'hyper-phosphorylation de CLK induits par la dérégulation du complexe PP2A/CKA/STRIP, les niveaux des transcrits de deux gènes cibles de la transcription par CLK, *vri* et *tim*, sont fortement diminués (données non montrées). Une baisse de la quantité des ARNm de *tim* a également pu être retrouvée dans les têtes de mouches exprimant le ARNi contre la sous-unité régulatrice *wdb*. Cependant, la dérégulation de *wdb* n'entraîne aucune différence quand à la quantité d'ARNm de *vri*. Cette différence entre les effets de CKA et WDB reflète les effets comportementaux des deux ARNi (*Cka*-ARNi donne un allongement de la période plus fort que *wdb*-ARNi). De plus, ces résultats mettent en évidence que le degré de phosphorylation de CLK (contrôlé par le complexe PP2A/CKA/STRIP) est plus important que sa quantité (contrôlé par PP2A/WDB) pour sa fonction de facteur de transcription.

En conclusion, au cours de ces travaux nous avons pu décrire deux complexes PP2A différents agissant sur la protéine CLK (Fig. 7, en haut): le complexe PP2A/CKA/STRIP contrôle la déphosphorylation de CLK – phénomène essentiel pour sa réactivation (Fig.7, en bas), tandis que PP2A/WDB affecte la stabilité de CLK indépendamment ou après l'action de PP2A/CKA.

De plus, nous avons trouvé que seule la dérégulation de la protéine STRIP, mais pas celle de CKA, affecte la stabilité de PER. Ces effets de stabilisation sont maintenus dans un contexte génétique où la protéine TIM n'est plus présente (*tim*⁰) et où PER n'est pas phosphorylé par la kinase DBT (mutants hypomorphe pour *dbt*) (résultats non montrés). STRIP posséderait ainsi des fonctions directes sur la stabilité de la protéine PER, et indépendantes de sa liaison avec CKA.

Enfin, le complexe enzymatique PP2A/CKA et la protéine STRIP font toutes partie d'un super-complexe appelé STRIPAK (striatin interacting kinase et phosphatase). En plus de PP2A/CKA et STRIP, ce complexe STRIPAK comprend des kinases de la famille Germinal Center kinase (Gck), appartenant au groupe Ste-20. Dans ce genre de super-complexes, phosphatases et kinases peuvent réguler mutuellement leur activité. Il reste donc à définir si le complexe STRIPAK contrôle le maintien d'une forme hypo-phosphorylée de CLK par l'inhibition (via déphosphorylation par la PP2A) d'une kinase de CLK associée à ce complexe, ou si CLK est directement déphosphorylé par la PP2A.

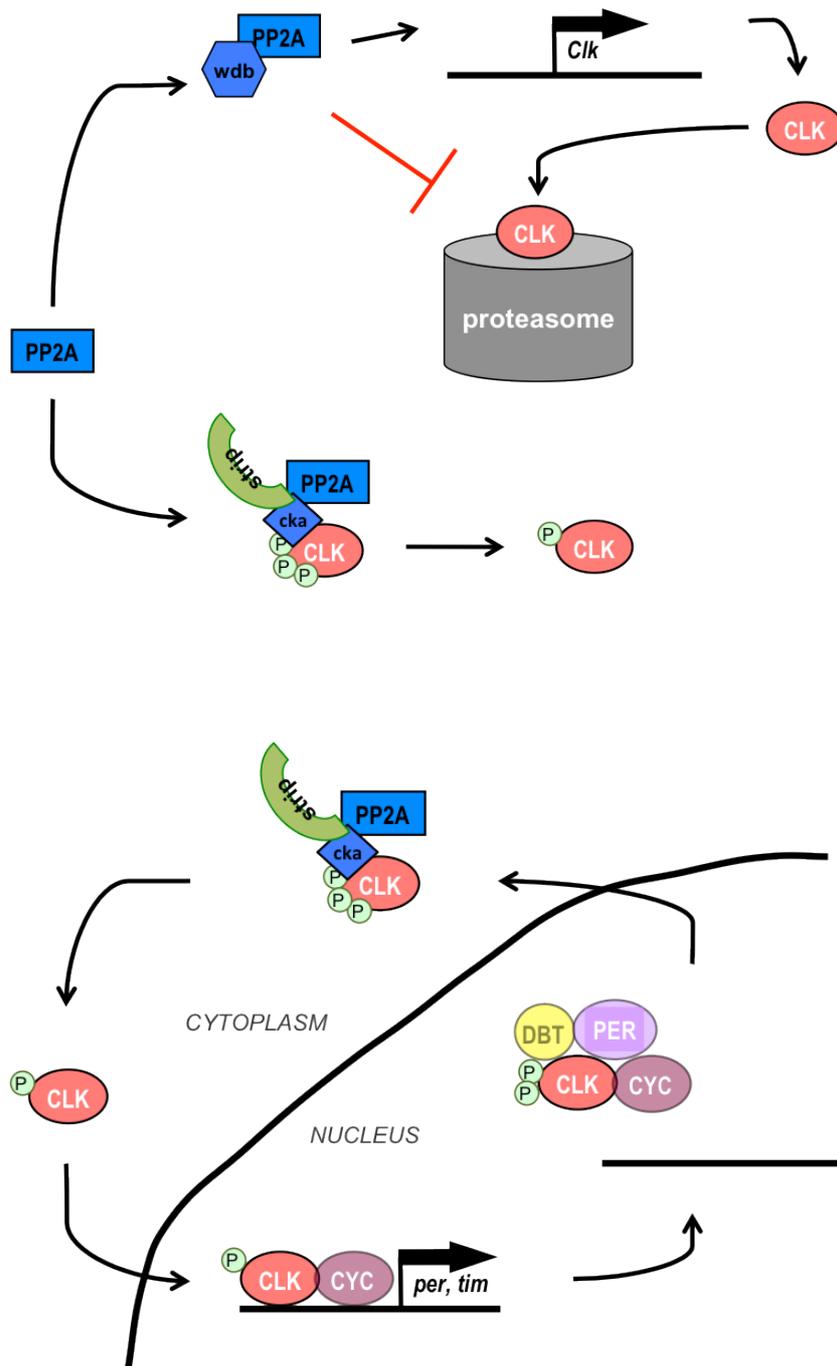


Figure 7 Schémas décrivant les rôles des deux complexes PP2A sur la protéine CLK

Deux complexes PP2A différents peuvent agir sur CLK : PP2A/Cka contrôle la déphosphorylation de CLK, et sa réactivation, tandis que le complexe PP2A/Wdb régule la quantité globale de CLK, selon des mécanismes encore inconnus (PP2A/Wdb pourrait réguler la transcription de *Clk* et/ou la stabilité de la protéine).

Pour conclure, cette étude a permis de décrire des nouveaux composants de la boucle moléculaire qui contrôlent les rythmes circadiens chez la drosophile.

La dégradation des protéines d'horloge est une étape essentielle pour un fonctionnement correct de la boucle moléculaire. Ainsi, depuis les années 2000, plusieurs enzymes E3 ubiquitine ligase ont été isolés ciblant PER, TIM et CLK pour la reconnaissance par le protéasome et leur dégradation. Toutefois, une seule enzyme ubiquitin-protease a été identifiée au sein de l'horloge moléculaire chez la drosophile, USP8. En déubiquitylant CLK, l'ubiquitin-specific peptidase 8 (USP8) régule l'activité de CLK à des moment précis du cycle (Luo et al., 2012). Pendant ces travaux, nous avons identifié une deuxième déubiquitinase, USP5, qui est impliquée dans le processus de dégradation des formes hyper-phosphorylées de PER présentes à la fin du cycle. Cette fonction peut être médiée par l'activité de désassemblage des polymères d'ubiquitine tel qu'il a été démontré chez l'orthologue d'USP5 chez l'homme et la levure.

En étudiant le rôle circadien de la protéine STRIP nous avons également pu comprendre une partie des modifications post-traductionnelles affectant la protéine CLK. Très peu est en effet actuellement connu sur les protéines modulant la stabilité et l'activité de la protéine CLK. Nous avons pu montrer que la phosphatase PP2A, en liaison avec sa sous-unité régulatrice CKA, contrôle la déphosphorylation de CLK au cours de la journée. Cette déphosphorylation est nécessaire pour rétablir le « stock » de CLK hypo-phosphorylé qui initie le nouveau cycle de transcription des gènes d'horloge au début de la nuit suivante. De façon indépendante et/ou consécutive à celle de la PP2A/CKA, un autre complexe PP2A, PP2A/WDB contrôle la stabilité de la protéine CLK, ainsi que celle des autres principaux partenaires de l'horloge moléculaire de la drosophile.

I. A BEHAVIORAL SCREEN USING TARGETED RNAi EXPRESSION IDENTIFIES USP5 AS A PUTATIVE *Drosophila* CORE CLOCK GENE

The *Drosophila* molecular clockwork is based on a transcriptional/translational feedback loop in which post-transcriptional modifications are crucial to maintain the pace of the clock. The analysis of the interacting partners that modify core clock components is of great interest to understand how the clock machinery sustains proper 24h-period rhythms and, possibly, sense different entraining signals.

Looking for new clock components, we set up a behavioral screen based on targeted expression of RNAi transgenes directed to half of the *Drosophila* genome. Fifty-four putative new clock genes have been identified, some of which have already been reported to function within the fruit fly molecular clock. Sylvina Bouleau (Post-doc) and Béatrice Martin (technician) primarily coordinate this project, with the help of few other members of the laboratory, including myself.

In the following pages, I will describe the screen and give evidence for the circadian role of one of the putative clock genes identified, the UBIQUITIN-SPECIFIC PROTEASE 5 (USP5). USP5 activity regulates the degradation of the PERIOD (PER) protein and, secondary, of the TIMELESS (TIM) protein. In addition, USP5 controls the stability of the CLOCK (CLK) protein.

Preliminary unpublished data describing the behavioral and molecular effects of another “positive” RNAi line (*CG17350*-RNAi) will be presented in ANNEXES (§ III).

1. Results

*a. Identification of new putative *Drosophila* clock genes*

In order to isolate new clock genes, a genome-wide behavioral screen was set using the Gal4-UAS technique to drive RNAi-mediated gene silencing specifically to clock neurons. The *gal1118* P-Gal4 transgene is strongly expressed in the PDF⁺ s-LN_v clock neurons (Blanchardon et al., 2001) that are known to control rhythmic locomotor behaviors in constant

dark conditions (Fig. 1A). We took advantage of a large-scale RNAi transgenic fly collection generated by the laboratory of Ryu Ueda (Mishima, Japan), which includes around 14,000 fly lines and covers roughly half of the *Drosophila* genome. *gal1118-Gal4* flies were crossed to flies bearing the UAS-RNAi transgene construct targeting a specific gene. Four young males (<3 days-old) from the resulting offspring (*gal1118-Gal4/+>UAS-RNAi/+*) were tested under constant dark (DD) conditions for seven days, after three days of entrainment in 12h:12h LD cycles (constant 23°C temperature) .

The screen was first validated by RNAi transgenes directed against known clock genes (Table 1). During the screen, 12,270 UAS-RNAi insertions, targeting 6,427 *Drosophila* genes (for the 91% of the genes tested, 2 independent UAS-RNAi insertions targeting a given gene were available) were analyzed. Iterative behavioral tests using a higher number of flies were repeated for RNAi lines inducing an altered locomotor behavior (3% of total RNAi insertions) (Fig. 1B). After removal of negative RNAi lines (giving no phenotype) and RNAi transgenes targeting housekeeping genes or ribosomal proteins, we finally selected 54 genes (Fig. 1B, Table 2). Anti-PDF staining in whole mounted brain of RNAi expressing flies was performed to assess the presence of healthy clock neurons. “Positive” putative clock genes were grouped according to the behavioral effects of their RNAi induction in period lengthening (76%) – for most of lines +1h, period shortening (2%) and arrhythmic (22%) (Fig. 1B and 1C).

b. usp5 downregulation lengthens the period of the fly rhythmic locomotor behaviors

Among the genes retained in our screen, downregulation of *CG12082/usp5* by RNAi expression induced a strong lengthening of the period of locomotor rhythms, and was selected for further studies. The cross between *gal1118-Gal4* flies with flies bearing the NIG-FLY *UAS-12082R2* transgene gave offspring with a locomotor period of 26h (2h longer than control flies bearing only the driver or the UAS-RNAi responder transgenes). Genetic outcrosses revealed the presence of two independent RNAi transgene insertions, one on the II chromosome (referred as *12082R2a*) and one on the III chromosome (*12082R2b*). *w*; *UAS-12082R2a*; *gal1118-Gal4/+* flies showed very long periods of 30h; while addition of an UAS-*dcr2* transgene increased the periodicity of *w*; *12082R2b/gal1118-Gal4* flies from 25h to 30h (*dicer2* (*dcr2*) overexpression enhances RNAi-mediated gene silencing). Long-period phenotypes were also observed when using a *pdf-Gal4* driver or a more broadly expressed *tim-Gal4* driver (Fig. 2 - Table 3).

A major problem when using RNAi transgenes is the occurrence of off-target effects. The *usp5*-RNAi long-period phenotype has been confirmed by the use of non-overlapping RNAi sequences from both the NIG-FLY and the VDRC collections (VDRC #17567 – Fig. 2, and #17568) (Table 3). This supported the idea that the period lengthening was specifically due to *usp5* downregulation. In addition, quantitative RT-PCR assay showed that *CG12082* mRNA quantities were efficiently dampened by expression of the *12082R2a* and *12082R2b* RNAi transgenes in *tim*-positive cells (Fig. S2). Moreover, *usp5* mRNA expression was not subjected to circadian regulation. Data were consistent with *usp5* being a clock gene involved in circadian period determination.

c. *usp5* downregulation alters PER and TIM oscillations in s-LN_vs

To start the characterization of *usp5* circadian function(s) we analyzed the oscillating profile of the PERIOD (PER) and the TIMLESS (TIM) proteins in the small-LN_v clock neurons of *usp5*-RNAi expressing flies (*UAS-dcr2/+; UAS-#17567/+; gal1118-Gal4/+*) and in control flies bearing only the driver transgene (*UAS-dcr2/+; +/+; gal1118-Gal4/+*) (Fig. 3A-3B). Flies were entrained to 12h light: 12h dark cycles and collected every 4h during the first two days of the following constant darkness (DD1 and DD2). In agreement with the strong period lengthening in locomotor behaviors, PER and TIM oscillations in s-LN_vs of *usp5*-RNAi flies were severely delayed with a slight decrease in protein quantities. Similar results were obtained with a different *usp5*-RNAi transgenic insertion (*12082R2a*) (Fig.S3).

In addition to altering clock protein cycling, *usp5*-RNAi expression in the *pdf*⁺ cells induced a significant increase in the cell body size of s-LN_v neurons (Fig. 3A-3C).

d. *Usp5* regulates degradation of clock proteins

usp5 depletion effects were then assayed by Western blot in total head protein extracts. The *tim*-Gal4 driver was used to express *usp5*-RNAi in all clock cells. Flies were collected every three hours during entraining 12h:12h LD conditions and on the first day after transfer in constant darkness. In DD conditions, PER and TIM were stabilized during daytime in extracts of *usp5*-RNAi expressing flies compared to controls (Fig. 4A). A more dramatic phenotype was observed on the PER protein. Indeed, low-migrating (hyper-phosphorylated) PER forms persisted in *usp5*-RNAi extracts at the end of the subjective day and at the

beginning of the subsequent night. At that time, hyper-phosphorylated PER had already undergone proteasome breakdown and no phospho-PER could be detected in control extracts, while newly synthesized, hypo-phosphorylated PER started to accumulate. This indicated that *usp5* depletion elicited a defect in the degradation program of the PER and TIM proteins. In LD conditions, proteins were also stabilized by *usp5*-RNAi expression; nevertheless, effects were weaker than in constant darkness. In particular, daytime degradation of TIM was very little affected whereas PER degradation was slowed down. Since TIM is the direct target of CRY-dependent light-induced degradation, we speculate that USP5 is not involved in the light-dependent degradation of (at least TIM) clock proteins but participate to their clock-controlled degradation.

The CLK protein was also affected upon *usp5*-RNAi expression, with an enrichment of hypo-phosphorylated CLK forms at the end of the day in DD and, to lesser extent, in LD (fig.4A). Hypo-phosphorylated CLK is the transcriptionally active form, while hyper-phosphorylated CLK accumulates at times when transcription is minimal. Quantitative RT-PCR assays were performed to quantify *per*, *tim* and *Clk* mRNAs in fly head extracts collected in DD. The *per* and *tim* transcripts showed dampened oscillations in *usp5*-RNAi flies compared to controls, with lower peak levels at CT15 (Fig 4B). The persistence of hyper-phosphorylated PER repressor at the beginning of the night likely limits CLK transactivation and proper transcription of the CLK target genes even if higher quantities of hypo-phosphorylated, transcriptionally active CLK is present. Lower *Clk* mRNA levels were also observed, possibly as a consequence of deregulation of *vri* and *pdp1ε* gene expression. *vri* and *pdp1ε* are CLK target genes that feed back to respectively repress and activate *Clk* transcription. For all the three clock components (PER, TIM, CLK), the comparison between mRNA and protein oscillations suggested that USP5 promoted protein stabilization.

e. USP5 directly controls degradation of the PER protein

To understand how USP5 might control the amounts of the different clock proteins, *usp5*-RNAi effects were analyzed by Western blot in a *per*⁰ or *tim*⁰ clock mutant backgrounds (Fig.4C and 4D). In such behaviorally arrhythmic flies, non-cycling low-phosphorylated TIM and CLK (*per*⁰) or PER and CLK (*tim*⁰) proteins are present at all time points. In *per*⁰ flies, *usp5* downregulation had no effects on the TIM or CLK levels, indicating that the higher abundance of these proteins in *usp5*-RNAi flies relied on the presence of the PER protein. In

contrast, *usp5* downregulation in *tim*⁰ flies induced higher levels of hypo-phosphorylated PER and CLK, suggesting that TIM was not required for USP5 function on the PER or CLK proteins. Data shown are consistent with a different mode of action for USP5 on the different clock proteins. USP5 might directly control stability of hyperphosphorylated PER while it might indirectly regulate maintaining of hyperphosphorylated TIM and (maybe) hypophosphorylated CLK.

2. Materials and Methods

Fly strains

gal1118-Gal4 (Blanchardon et al., 2001), *pdf-Gal4* (Kaneko and Hall, 2000) and *tim-Gal4* (Kaneko, 1998) have been already reported. *usp5*-RNAi lines come from the Japanese NIG-FLY stock (*w;12082R2a;12082R2b*) or the Austrian VDRC fly stock (#17567 and #17568). *UAS-dcr2* flies are also from the VDRC fly stock.

RNAi screen setup

Gene inactivation by RNAi expression was performed using an RNAi transgenic fly library (NIG-FLY stock from R. Ueda, Genetic Strains Research Center) covering about 50% of the predicted coding sequences of the *Drosophila* genome. About 12,400 lines were tested, that correspond to more than 6,000 predicted genes (there were mostly two insertion lines for one gene). For each cross, the locomotor activity of 4 male flies was monitored for 7 days in constant darkness at 23°C after two days of 12h: 12h light-dark entrainment. *UAS-RNAi*-bearing transgenic males were crossed with *gal1118-Gal4* virgin females (A *UAS-HShid* insertion associated to the Y chromosome was used to get rid of males from the driver line after heat-shock treatment). Crosses with the *CKIIa*-RNAi line (17520-R2 line from the NIG-FLY databank) were used as a behavioral control (*w; UAS-CKIIa-RNAi/+; gal1118-Gal4/+* flies behave with a period of 26h). RNAi-expressing flies showing a modification in their period length of at least 1h compared to that of control flies, as well as flies showing at least 50% of arrhythmicity were selected. As such retained RNAi lines were then behaviorally tested a second time increasing the number of tested flies to 12. Eventually, still positive lines were tested again at 25°C with 16 flies to confirm the phenotype. Positive RNAi insertions were also checked for their chromosomal localization by genetic crosses. Unaffected morphology

of clock neurons was assessed by anti-PDF immunostaining of whole-mounted RNAi-expressing fly brains.

Behavioral analysis

Experiments were carried out with 1 to 7-day-old males at 25 °C in *Drosophila* activity monitors (TriKinetics) as previously described (Klarsfeld et al., 2003). For DD analysis, flies were first entrained in 12h: 12h LD cycles during 2 to 4 days before transfer to DD, and data were analysed for 7 days from the second day in DD. Data analysis was done with the FaasX software, which is derived from the Brandeis Rhythm Package and is available on request. Rhythmic flies were defined by X^2 periodogram analysis with the following criteria (filter on): power ≥ 20 , width ≥ 2 h, with no selection on period value. Power and width are the height and width of the periodogram peak, respectively, and give the significance of the calculated period. Mean daily activity (number of events per 0.5 h) is calculated over the whole period in DD conditions. All behavioural experiments were reproduced two or three times with very similar results.

Flies entrainment and collection for biochemical analyses

Flies were entrained during at least 3 days in LD conditions at 25°C before being collected for biochemical analyses. For Western blots and quantitative RT-PCR assays flies were collected every 3 hours while for dissections collection of flies was every 4 hours.

Immunolabelings

All experiments were done on whole-mounted brains as previously described (Blanchardon et al., 2001). Mouse anti-PDF antiserum (Developmental Studies Hybridoma Bank) was used at 1:50,000. Rabbit anti-PER antiserum (Stanewsky et al., 1997) was used at 1:15,000. Rat anti-TIM antiserum (Grima et al., 2002) was used at 1:10,000. Secondary antibodies were Alexa647 anti-rat at 1:5,000; Alexa488 anti-mouse 1:2,000 and Alexa594 anti-rabbit at 1:5,000 (Invitrogen-Life Technologies). Fluorescence signals were analysed with a Zeiss Axio Imager Z1 microscope equipped with an ApoTome system and a digital camera (AxioCam MR). Fluorescence intensity was quantified from digital images with the ImageJ software. We applied the formula: $I = 100(S - B)/B$, which gives the fluorescence percentage above background (where S is fluorescence intensity and B is average intensity of the region adjacent to the positive cell).

Western blots

Protein extracts were made from 30 to 40 heads for each time point. Frozen heads were homogenized in 150µl ice-cold RBS buffer (Yu et al., 2006) supplied with protease inhibitors (Roche) and phosphatase inhibitors cocktails 1 (0,5%) and 2 (1%) (Sigma) and βGP 2mM. Extracts were quantified using a Bradford (BioRad) protein assay. For SDS-PAGE, 25 to 100 µg of protein were separated on 3-8% Tris-Acetate gels or 4% Tris-Glycine gels (Invitrogen-Life Technologies) and transferred to PVDF membranes. Primary antibodies: rabbit anti-PER 1:10,000 (Stanewsky et al., 1997); goat anti-CLK sc-27070 1:1,000 (Santa Cruz biotechnology, USA); rat anti-TIM 1:2,000 (Grima et al., 2002). All secondary antibodies are from Santa Cruz Biotechnology.

Quantitative RT-PCR

RNA extraction is done with 30 to 50 fly heads with the SV Total RNA Isolation System (Promega). 31µl of the RNA extract is used to synthesize cDNA (Recombinant RNasin Ribonuclease Inhibitor, Oligo(dT)15 Primer, dNTP and the M-MLV Reverse Transcriptase RNase H Minus were from Promega). Quantitative RT-PCR was performed with a Lightcycler (Roche) using the SYBR green detection protocol as described previously (Grima et al., 2002).

Primer sequences used for RT-PCR experiments are as follow:

Gene	Orientation	Sequence (5'-3')	PCR fragment size
<i>tubulin</i>	sense antisense	TCCTTGTCGCGTGTGAAACA CCGAACGAGTGAAGATGAG	464
<i>usp5</i> <i>CG12082</i>	sense antisense	CAGCGACCCGGCCAACGACTT GAACTCCCGCACCTCATCCA	396
<i>period</i>	sense antisense	ACCCGCATCCTTCGCTTTTCTA CA GGGCAGGAGTGGTGACCGAGT GGA	219
<i>timeless</i>	sense antisense	AGTTGGTCATGCGCAGCAAAT G TCTTTTCGTACACAGATGCCA	448
<i>Clock</i>	sense antisense	CCAAATAAATCCACTGAAAAC AT GAGAGGCACCATTTTCGGAGT AC	367
<i>Pdp1ε</i>	sense antisense	GCGGCAACTGGTAATG ATTCCTGCCTGAGCT	217
<i>vriille</i>	sense antisense	CCGCCGGGCAAACCTCTATC GCGACTGGGCCATCATTCC	298

3. Figure Legends

Table1: *gal1118-Gal4>UAS-RNAi*–mediated downregulation of known clock genes confirms induction of altered locomotor behaviors.

Flies bearing one copy of the *gal1118-Gal4* driver and one copy of the *UAS-RNAi* responder targeting each known clock gene were entrained for 2 days in standard 12h light-12h dark (LD) conditions and then tested for 7 days in constant darkness (at constant 23°C temperature). Behavioral analyzes are performed on the last 6 days of constant conditions. The percentage of rhythmic flies (% Rhythmic) among those that survive until the end of the experiment (N) is shown, together with the period (Tau) displayed by the rhythmic flies. The power value indicates the robustness of the rhythm. sem: standard error of the mean.

Table2: List of the 54 “positive” putative clock genes.

The table lists the CG number and gene symbol for each of the 54 positive genes retained in the screen. The transgenic NIG-FLY line(s) used to downregulate each gene is indicated. *gal1118-Gal4/+;UAS-RNAi/+* flies were entrained in standard LD conditions and then tested in constant darkness for 7 days, at constant 25°C temperature. An asterisk marks genotypes tested at constant 23°C instead of 25°C. Data are referred to the last 6 days of constant conditions. For each genotype, the number of flies that survive until the end of the experiment (N) and the percentage of rhythmic flies among them are shown, together with the period (Tau) of their rhythmic behaviors. The power indicates the robustness of the rhythm. RNAi insertions that did not alter the rhythmicity of locomotor behaviors of *gal1118-Gal4* expressing flies are shown into brackets.

Table 3: *usp5-RNAi* expression in clock neurons induces long period rhythms.

Behavioural analysis in free running conditions (7 days) of control flies or flies expressing *usp5-RNAi* in clock neurons using the *gal1118-Gal4* (A and C), the *pdf-Gal4* and *tim-Gal4* (B) drivers. *12082R2a* and *12082R2b* are two independent insertions of an *usp5-RNAi* transgene (*12082R2*) from the NIG-FLY collection (A and B). #17567 and #17568 are two independent insertions of a same *usp5-RNAi* transgene from the VDRC collection (C). *dicer2* (*dcr2*) is a component of the RNA interference machinery which overexpression is known to enhance RNAi-mediated gene silencing. In bold, genotypes expressing *usp5-RNAi*. Tau: period value; sem: standard error of the mean.

Fig. 1: A behavioral screen targeting RNAi-mediated downregulation of half of the *Drosophila* genome in s-LN_vs clock neurons isolated new putative clock genes.

(A) The *gal1118-Gal4* driver line allows a strong UAS-*gfp* responder signal in the small-LN_v clock neurons. (B) Diagrams representing the score of positives genes retained through each step of the screen and the period range of the altered rhythmic phenotypes induced by “positive” RNAi expression (C).

Fig. 2: *usp5*-RNAi expression induces long period phenotypes.

Actograms showing average locomotor behaviors of N flies expressing *usp5*-RNAi in s-LN_vs (*gal1118-Gal4* driver) and, broader, in all *tim*⁺ clock cells (*tim-Gal4* driver). Flies were entrained for 2-3 days in 12h light-12h dark conditions, followed by constant darkness, at constant temperature of 25°C. The first 8 days of DD are shown. N: the number of flies tested.

Fig. 3: Delayed PER and TIM protein oscillations in s-LN_v neurons of *usp5*-RNAi flies

Anti-PER and anti-TIM immunolabeling of s-LN_v clock neurons in *usp5*-RNAi expressing flies (*UAS-dcr2/+; UAS-#17567/+; gal1118-Gal4/+*) and controls (*UAS-dcr2/+; gal1118-Gal4/+*) during the first two days of constant darkness following 12h:12h LD entrainment. (A) Image showing PER and TIM immunostaining in s-LN_vs of adult *usp5*-RNAi flies and corresponding controls at CT28, CT36 and CT44. Scale bar represent 25 μm. (B) Curves showing PER and TIM proteins concentration every four hours. Arrows indicate the three time points pictured in (A). *pdf*⁺ s-LN_vs labeling from 8 to 10 brain hemispheres was quantified for each time point and values averaged. Error bars indicate standard error of the mean (sem). AU: arbitrary units. Black and grey boxes indicate subjective night and day, respectively. (C) Anti-PDF, anti-PER and anti-TIM staining in brains of adult *usp5*-RNAi flies and corresponding controls at ZT0. The four s-LN_v neurons are marked by an asterisk. *usp5*-RNAi expression promotes s-LN_vs nuclear overgrowth. Scale bar represent 20 μm.

Fig. 4: *usp5*-RNAi expression alters the molecular oscillations of PER, TIM and CLK proteins

(A) Western blot of *usp5*-RNAi and control fly head extracts collected every 3 hours during standard LD (12h light-12h dark) and on the first day of DD conditions. PER, TIM and CLK molecular profiles are affected by *usp5*-RNAi. Anti-DBT blot is used as loading control. Data are representative of three independent experiments. (B) *Clk*, *per* and *tim* mRNA levels in

control and *usp5*-RNAi flies. Data are obtained using quantitative RT-PCR assays in heads of flies collected every 3 hours during DD1. Curves represent average values from two independent experiments – except for the control value at CT9 (only one replicata). Crosses indicate the value obtained for each serie. Black, grey and white boxes indicate night or subjective night, subjective day, and day, respectively. C: control: *w; tim-Gal4/+*; R: *usp5*-RNAi: *w; tim-Gal4/12082R2a; 12082R2b/+*. ZT: zeitgeber time (LD conditions). CT: circadian time (DD conditions). (C-D) Western blot of head extracts of flies collected at DD1 in a wild-type (WT: *per*⁺, *tim*⁺) and *per*⁰ (C) or *tim*⁰ (D) background. Both null mutations cause arrhythmic behaviors and lost of protein oscillations. PER is stabilized in a *tim* null background while TIM is not in a *per*⁰ background. WT: wild-type: *w; ;*. C: control: *per*⁰, *w; tim-Gal4/+*; (C) and *w; tim*⁰, *tim-Gal4/tim*⁰; (D). R: *usp5*-RNAi: *per*⁰, *w; tim-Gal4/12082R2a; 12082R2b/+* (C) and *w; tim*⁰, *tim-Gal4/tim*⁰, *12082R2a; 12082R2b/+* (D). CT: circadian time.

Supp. Fig. S1: RNAi expression reduces endogenous *usp5* mRNA quantities.

Quantitative RT-PCR of fly head extracts during LD and DD1 shows a reduction in *usp5* mRNA quantities upon RNA expression. Data also indicate that *usp5* transcription does not oscillate during the 24 hours.

Supp. Fig. S2: Delayed PER and TIM protein oscillations in s-LNv neurons of *usp5*-RNAi flies (NIG-FLY RNAi line)

Anti-PER and anti-TIM immunolabeling signal of s-LNv clock neurons in *usp5*-RNAi expressing flies (*w; UAS-12082R2a; gal1118-Gal4/+*) and controls (*w; ; gal1118-Gal4/+*) during two days starting from the last night of 12h:12h LD entrainment followed by constant darkness conditions. Flies were dissected every four hours. *pdf*⁺ s-LN_vs labeling from 8 to 10 brain hemispheres was quantified for each time point and values averaged. Error bars indicate standard error of the mean (sem). AU: arbitrary units. Black boxes indicate night and subjective night, while grey boxes indicates subjective days respectively.

Table 1

Gene name	Genotype (RNAi line)	N	% Rhythmic	Tau (h) ± sem	Power ± sem
<i>period</i>	w;;UAS-2647R-1/ <i>gal1118-Gal4</i>	16	100	24.5 ± 0.2	84 ± 6
	w;;UAS-2647R-2/ <i>gal1118-Gal4</i>	16	94	24.4 ± 0.2	76 ± 8
<i>timeless</i>	w;;UAS-3234R-1/ <i>gal1118-Gal4</i>	15	80	25.0 ± 0.3	99 ± 7
<i>doubletime</i>	w;;UAS-2048R-1/ <i>gal1118-Gal4</i>	15	100	25.0 ± 0.2	98 ± 9
<i>cycle</i>	w;;UAS-8727R-1/ <i>gal1118-Gal4</i>	16	63	23.8 ± 0.8	44 ± 4
<i>clockwork orange</i>	w;;UAS-17100R-2/ <i>gal1118-Gal4</i>	16	100	25.5 ± 0.1	122 ± 8
<i>Casein kinase II α</i>	w;UAS-17520R-1/+; <i>gal1118-Gal4</i> /+	15	93	25.6 ± 0.1	142 ± 4
	w;;UAS-17520R-2/ <i>gal1118-Gal4</i>	16	100	25.3 ± 0.1	139 ± 4
<i>Casein kinase II β</i>	w;UAS-15224R-1/+; <i>gal1118-Gal4</i> /+	16	100	25.4 ± 0.0	149 ± 4
<i>shaggy</i>	w;;UAS-2621R-3/ <i>gal1118-Gal4</i>	7	100	24.5 ± 0.3	119 ± 8
<i>Clock</i>	w;;UAS-7391R-3/ <i>gal1118-Gal4</i>	16	93.8	25.7 ± 0.1	83 ± 9
Control	w;; <i>gal1118-Gal4</i> /+	16	100	24.0 ± 0.1	132 ± 5

Table 2

CG (name/symbol)	RNAi lines	NumAlive	%Rhythmic	RNAi	
				Tau	Power
15610 (<i>muscle wasted / mute</i>)	15610R-3* (15610R-4)	16	100	25.2 +/- 0.2	167 +/-7
3929 (<i>deltex / dx</i>)	3929R-1*	16	100	26 +/- 0.1	138 +/-16
	3929R-2*	16	100	25.6 +/- 0.1	182 +/-6
4214 (<i>syntaxin5 / Syx5</i>)	4214R-4*	16	69	22.5 +/- 0.5	84 +/-8
	(4214R-1)				
5216 (<i>Sir2</i>)	5216R-1w* (5216R-2)	16	100	25.6 +/- 0.1	196 +/-3
11888 (<i>Rpn2</i>)	11888R-1*	14	86	29.4 +/- 1.8	65 +/-8
	11888R-3*	16	81	25.5 +/- 0.7	72 +/-10
15319 (<i>nejire / nej</i>)	15319R-2	15	53	27.3 +/- 0.5	49 +/-7
	15319R-3	16	94	28.4 +/- 0.3	90 +/-9
12701 (<i>vielfaltig / vfi</i>)	12701R-1*	12	100	26.6 +/- 0.2	129 +/-7
	(12701R-2)				
12082 (<i>drosophila ubiquitine protease 5 / dUSP5</i>)	12082R-2* (12082R-1)	12	100	26.1 +/- 0.2	147 +/-10
1828 (<i>dre4</i>)	1828R-2*	24	88	23.7 +/- 0.2	60 +/-5
8264 (<i>Bx42</i>)	8264R-1*	11	73	23.2 +/- 0.4	58 +/-14
	8264R-3*	26	58	25.2 +/- 1.2	49 +/-7
11526	11526R-1* (11526R-2)	16	100	25.7 +/- 0.1	156 +/-7
6292 (<i>Cyclin T / CycT</i>)	6292R-1* (6292R-3)	23	96	25.8 +/- 0.3	134 +/-13
7033	7033R-1w*	16	100	25.3 +/- 0.1	122 +/-6
	7033R-3*	9	100	26.9 +/- 0.2	103 +/-11
7467 (<i>osa</i>)	7467R-1*	16	100	25.4 +/- 0.1	127 +/-8
	(7467R-3)				
5374 (<i>Tcp1 like / T-cp1</i>)	5374R-2*	16	100	25.5 +/- 0.1	163 +/-7
31164 (<i>Ionotropic receptor 94a / Ir94a</i>)	31164R-3*	16	100	25.9 +/- 0.1	166 +/-6
	(31164R-1)				
7974	7974R-1* (7974R-3)	16	88	26.3 +/- 0.2	105 +/-8
4170 (<i>vasa intronic gene / vig</i>)	4170R-3 (4170R-1)	15	100	25.4 +/- 0.1	161 +/-13
17350	17350R-2	16	94	26.3 +/- 0.1	137 +/-7
	(17350R-1)				
3025 (<i>males absent on the first/ mof</i>)	3025R-2	16	100	24.8 +/- 0.1	165 +/-10
	3025R-3	14	100	25.5 +/- 0.1	130 +/-11
11811	11811R-3;;	16	100	24.7 +/- 0.1	184 +/-5
	(11811R-7)				
4153 (<i>Eukaryotic initiation factor 2β / eIF-2β</i>)	;;4153R-2	16	88	23.4 +/- 0.7	65 +/-11
	;4153R-8	14	71	22.9 +/- 0.2	87 +/-16
5495 (<i>thioredoxin-like / Txi</i>)	;;5495R-1	16	100	26.2 +/- 0.1	160 +/-13
	;;5495R-2	15	100	25.6 +/- 0.1	164 +/-7
13387 (<i>embargoed / emb</i>)	;;13387R-1	15	100	25.1 +/- 0.1	151 +/-9
	;13387R-4	16	100	24.9 +/- 0.1	155 +/-6
16903	16903R8 (16903R2)	16	100	25.2 +/- 0.1	181 +/-7
17282	17282R-3 (17282R-2)	15	100	25 +/- 0.1	152 +/-9
7776 (<i>Enhancer of Polycomb / E(Pc)</i>)	;;7776R-1	15	100	23.8 +/- 0.2	90 +/-7
	;7776R-2	9	89	23.6 +/- 0.3	63 +/-7
13222 (<i>Cuticular protein 47Ee / Cpr47Ee</i>)	13222R-4 (13222R-1)	11	100	25.4 +/- 0.1	197 +/-4
3319 (<i>cyclin-depd kinase 7 / Cdk7</i>)	3319R-2 (3319R-1)	16	94	24.9 +/- 0.1	155 +/-8
10385 (<i>male-specific lethal 1 / msl-1</i>)	10385R-1	16	100	26 +/- 0.1	152 +/-10
	10385R-3	16	100	26 +/- 0.1	149 +/-7
11396	11396Rb-2 (11396Rb-1)	16	100	24.7 +/- 0.9	61 +/-4
10693 (<i>slowpoke / slo</i>)	10693R-2 (10693R-1)	16	100	24.9 +/- 0.1	175 +/-7
15247	15247R-1	16	100	25.7 +/- 0.3	78 +/-6
	(15247R-4)				
11282 (<i>capricious / caps</i>)	11282Rb-1	16	100	24.7 +/- 0.9	136 +/-6
	(11282Rb-2)				
7110	7110R-1 (7110R-2)	16	100	25.3 +/- 0.1	165 +/-8
9650	9650R-1 (9650R-4)	14	100	26.1 +/- 0.5	77 +/-6
8439 (<i>T-complex Chaperonin 5 / Cct5</i>)	8439R-1 (8439R-4)	16	100	24.9 +/- 0.1	166 +/-6
5519 (<i>Prp19</i>)	5519R-4 (5519R-1)	16	100	25.4 +/- 0.1	158 +/-10
14884 (<i>COP9 complex homolog subunit 5 / CSN5</i>)	14884R-3 (14884R-1)	16	94	25.1 +/- 0.1	183 +/-5
3051 (<i>SNF1A/AMP-activated protein kinase / SNF1A</i>)	3051R-1 (3051R-2)	15	87	25 +/- 0.1	151 +/-12
5684 (<i>Pop2</i>)	5684R-4	16	100	24.7 +/- 0.1	154 +/-6
12301	12301R-2 (12301R-1)	16	75	25.3 +/- 0.3	77 +/-12
15507 (<i>kayak / kay</i>)	15507R-2 (15507R-4)	14	100	25.4 +/- 0.1	193 +/-5
12164	12164R-1 (12164R-2)	16	100	24.9 +/- 0.1	181 +/-4
11799 (<i>forkhead domain 68A / fd68A</i>)	11799R-4 (11799R-1)	16	100	25.8 +/- 0.1	191 +/-3
5208 (<i>Protein associated with topo II related - 1 / Patr-1</i>)	5208R-3 (5208R-1)	16	100	24.9 +/- 0.1	185 +/-7
5643 (<i>widerborst / wdb</i>)	5643R-4 (5643R-3)	16	100	25 +/- 0.1	165 +/-7
10623	10623R4 (10623R3)	5	40	24.3 +/- 0.3	57 +/-3
1884 (<i>Not1</i>)	1884R-1 (1884R-3)	16	50	23.2 +/- 0.2	70 +/-12
4586	4586R-2	16	100	25.8 +/- 0.1	183 +/-5
	4586R-3	15	100	2.8 +/- 0.1	181 +/-7
3806 (<i>eIF2B-e</i>)	3806R-3	16	100	25.7 +/- 0.1	147 +/-5
	3806R-5	16	100	25.7 +/- 0.1	148 +/-10
4952 (<i>dachshund</i>)	4952R-2	14	100	25.1 +/- 0.1	160 +/-10
	(4952R-4)				
1782 (<i>Ubiquitin activating enzyme 1 / Uba1</i>)	1782R-1 (1782R-2)	15	100	25.3 +/- 0.1	116 +/-10
4999 (<i>Tetraspanin 66E / Tsp66</i>)	4999R-2 (4999R-1)	14	100	24.8 +/- 1.0	175 +/-6
	;;gal1118/+	8	100	23.9 +/- 0.1	155 +/-9
	;;gal1118/+	12	100	24.2 +/- 0.1	173 +/-7

Table 3**A**

Genotype	N	%R	Tau	sem	Power	sem
<i>w;;gal1118-Gal4/+</i>	12	100.0	24.2	0.09	172.7	6.77
<i>w;UAS-12082R2a/+;UAS-12082R2b/+</i>	16	100.0	23.9	0.11	139.5	10.65
<i>w,UAS-dcr2/+;;gal1118-Gal4/+</i>	16	93.8	24.6	0.08	145.9	12.79
<i>w,UAS-dcr2/+;;</i>	16	100.0	23.9	0.08	142.7	11.22
<i>w;UAS-12082R2a/+;UAS-12082R2b/gal1118-Gal4</i>	12	100.0	26.1	0.19	147.0	10.16
<i>w;UAS-12082R2a/+;Gal1118/+</i>	16	100.0	24.5	0.07	175.8	7.17
<i>w;UAS-12082R2a;Gal1118/+</i>	17	88.2	30.0	0.41	67.1	6.87
<i>w;;UAS-12082R2b/Gal1118</i>	16	93.8	25.3	0.10	166.8	7.55
<i>w;UAS-dcr2/+;UAS-12082R2b/Gal1118</i>	21	100.0	30.3	0.16	83.0	6.29

B

Genotype	N	%R	Tau	sem	Power	sem
<i>w;pdf-Gal4/+;</i>	15	100.0	24.0	0.10	180.1	6.70
<i>w;pdf-Gal4/UAS-12082R2a;UAS-12082R2b/+</i>	16	100.0	25.5	0.09	167.4	6.08
<i>w;tim-Gal4/+;</i>	16	100.0	24.8	0.18	132.4	12.25
<i>w;tim-Gal4/UAS-12082R2a;UAS-12082R2b/+</i>	22	95.5	26.2	0.21	130.2	10.22

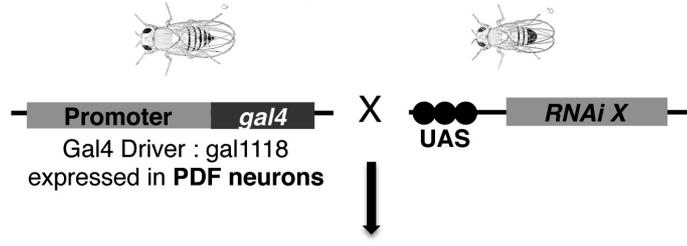
C

Genotype	N	%R	Tau	sem	Power	sem
<i>w;UAS-#17567/+;Gal1118/+</i>	16	100.0	24.0	0.06	191.9	4.61
<i>UAS-dcr2/+;UAS-#17567/+;Gal1118/+</i>	16	100.0	28.5	0.19	123.4	6.98
<i>w;UAS-#17568/+;Gal1118/+</i>	16	100.0	24.3	0.08	183.5	4.78
<i>UAS-dcr2/+;UAS-#17568/+;Gal1118/+</i>	16	100.0	28.9	0.14	112.7	5.03

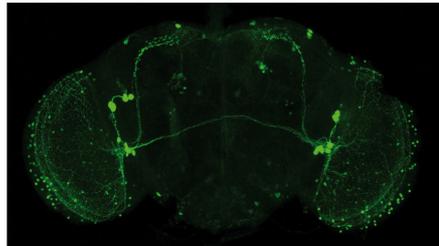
Figure 1

A

UAS-gal4 system

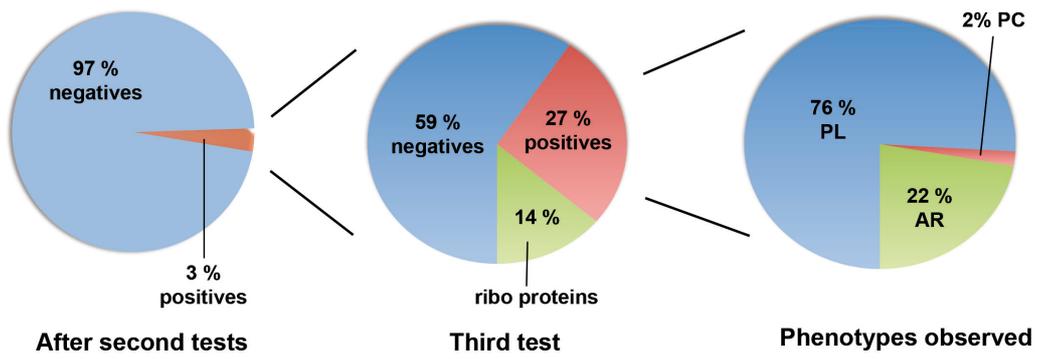


The RNAi is mostly expressed in PDF neurons



W;UASGFP/+;gal1118/+

B



C

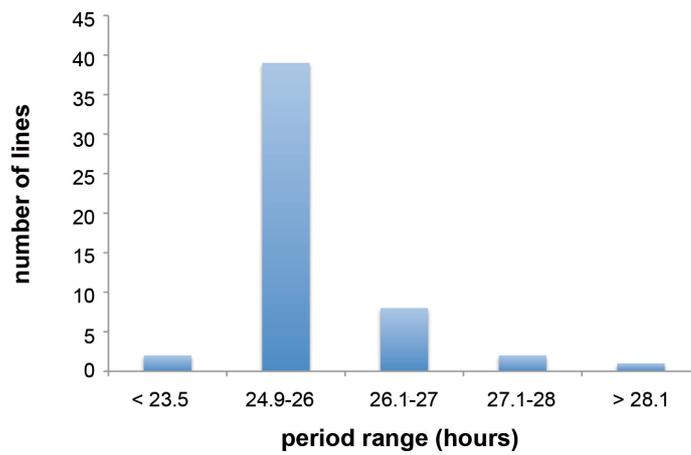


Figure 2

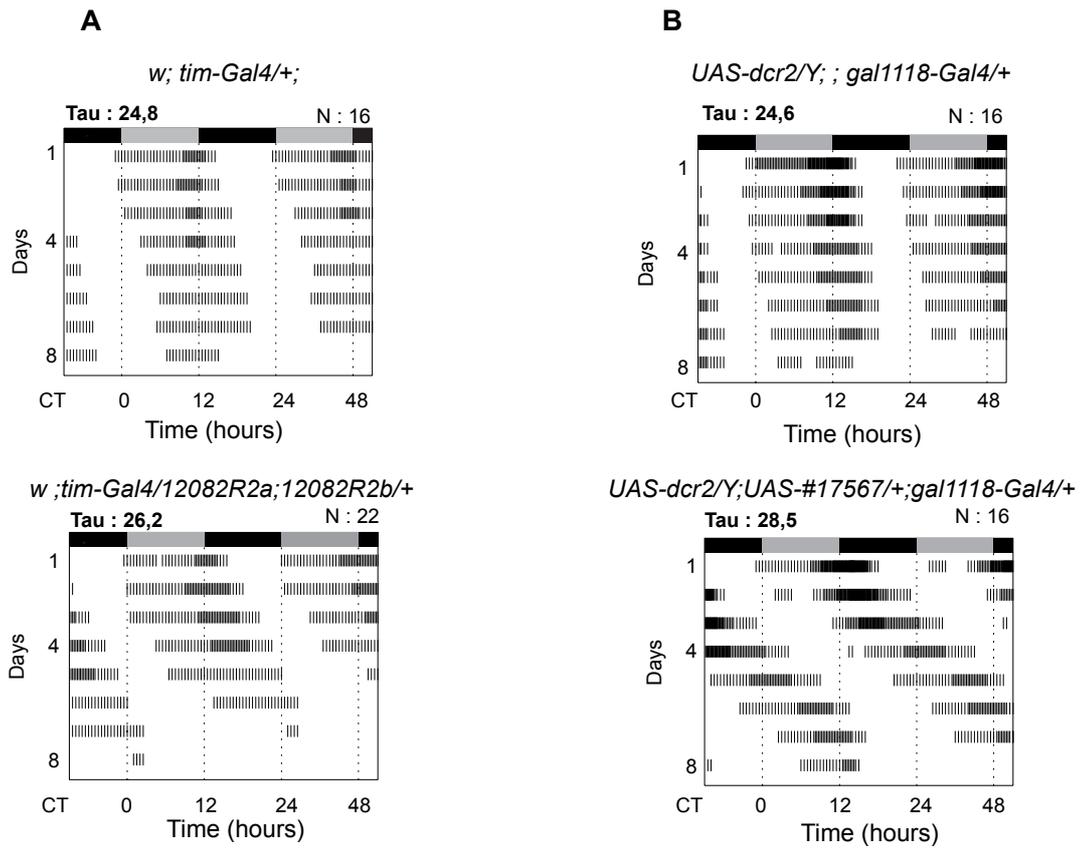


Figure 3

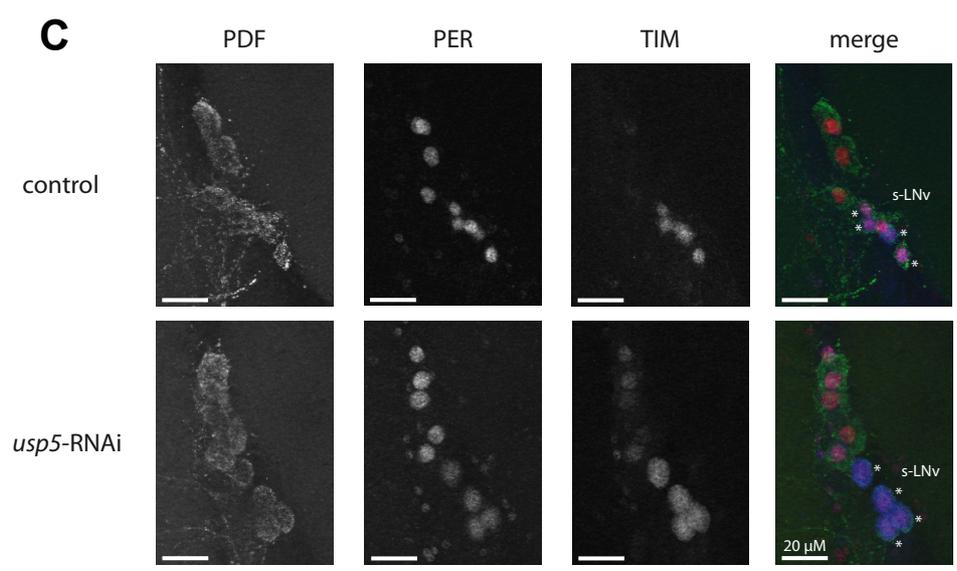
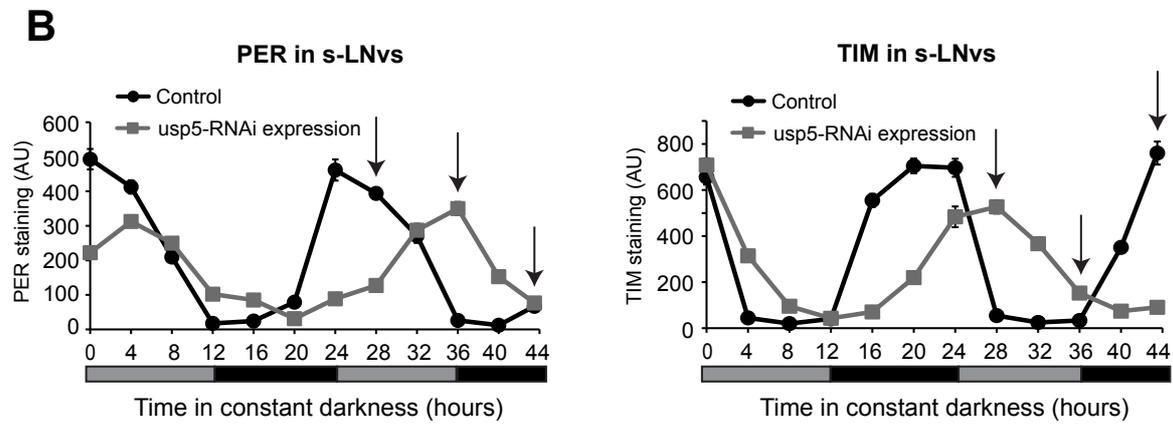
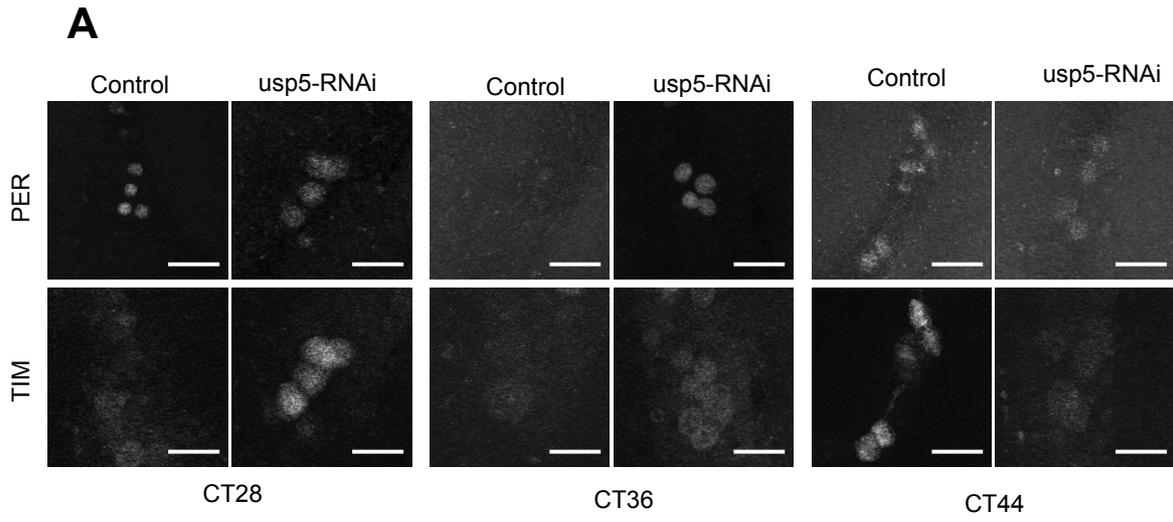
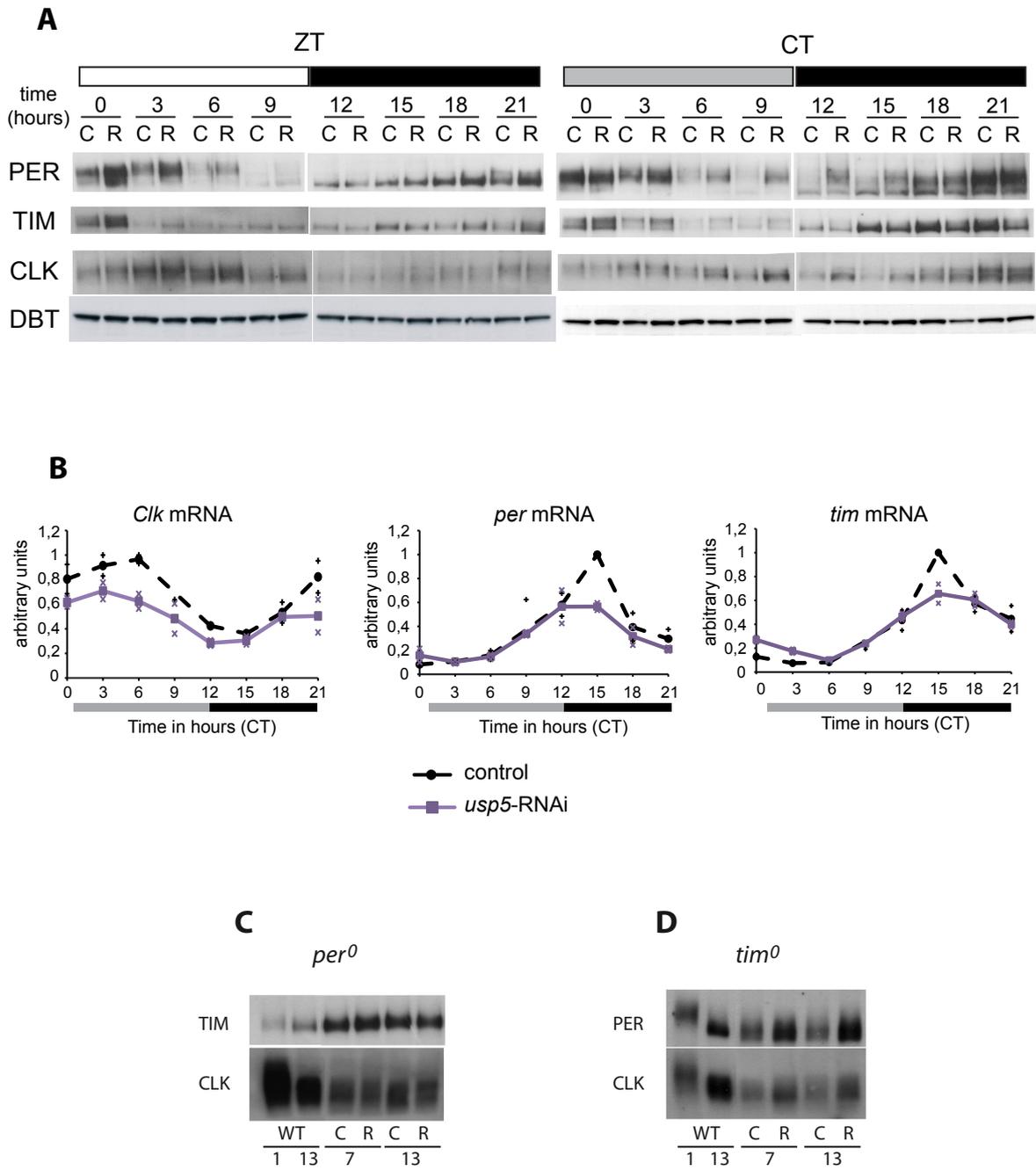
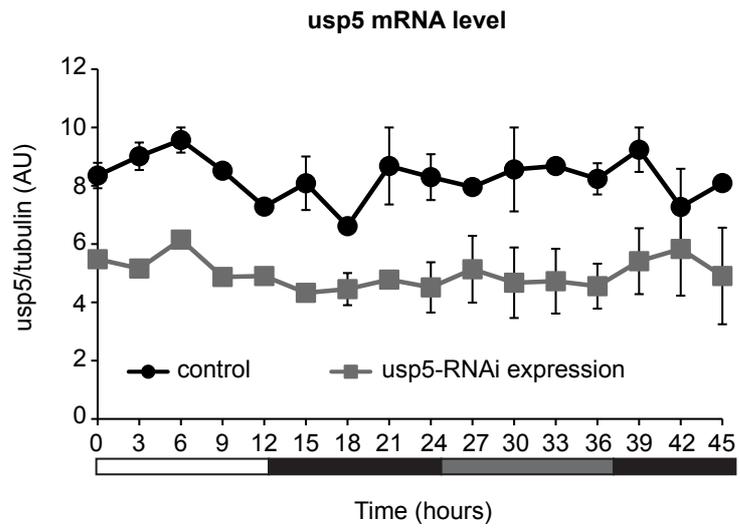


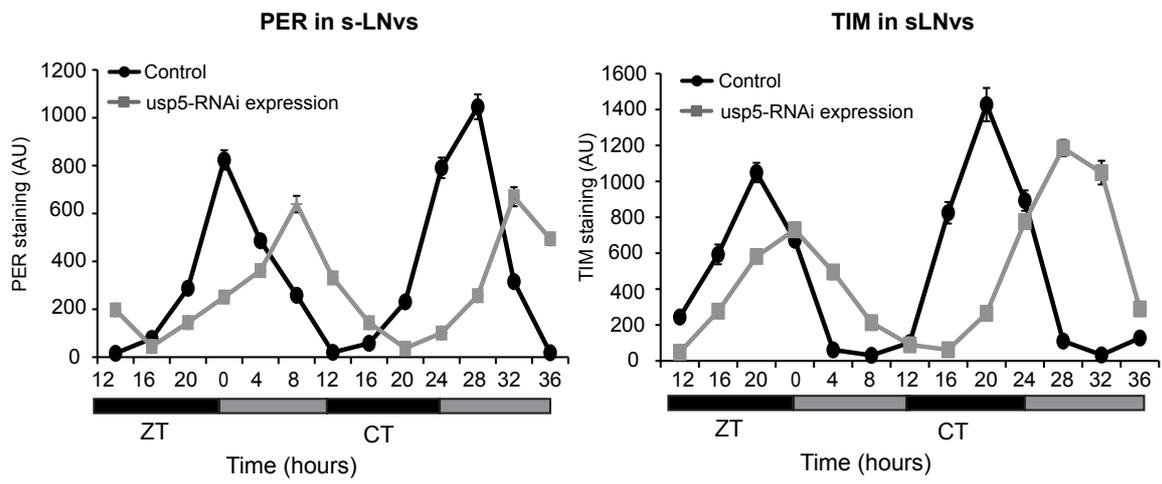
Figure 4



Supplemental Figure S1



Supplemental Figure S2



4. Discussion

*a. RNAi-based pan-genomic screen allows the isolation of new *Drosophila* clock components*

Several studies have highlighted the role of post-translational modifications in maintaining the pace of the circadian clock close to 24h. Indeed, deregulation of some kinase, phosphatase and ubiquitin ligase activities affects the length of the molecular cycle and the period of output behaviors, eventually causing arrhythmicity. In this study, we set up a pan-genomic behavioral screen using targeted RNAi-mediated gene knockdown.

RNA interference (RNAi) is a powerful and effective method used to downregulate gene expression in various animal models, including *Drosophila*. The RNAi method was coupled to the Gal4-UAS technique to target gene silencing uniquely to clock neurons (see ANNEXES § II.2). This allowed bypassing of the possible developmental lethality caused by a more robust gene knockdown. In addition, RNAi-based approaches allow for gene dosage studies and may show subtle effects that would be missed in null mutants or nearly complete gene knockdown. However, while powerful, RNAi technology also has some flaws, such as off-target effects. Off-target effects depend on the sequence similarity between the RNAi molecule and one or more genes other than the target. Effects of RNAi expression would then recapitulate downregulation of a gene different from that of interest (see ANNEXES § III). The use of multiple/non-overlapping RNAi sequences and independent gene silencing tools (such as P element insertions and mutants) are therefore required to corroborate “on target” effects from preliminary data obtained by the use of RNAi.

Based on the behavioral effects of their RNAi-mediated downregulation we selected 54 putative clock genes. The large majority of the selected genes encode for protein of poorly studied or unknown functions. However, among those genes is the *nejire* (CBP) transcriptional regulator (*nej*-RNAi induces rhythmic 26h period behaviors), whose circadian functions were recently characterized (Hung et al., 2007). The *slowpoke* (*slo*) gene, also isolated in this screen, codes for a calcium-activated potassium channel that is required for circadian rhythmicity (Ceriani et al., 2002). In addition, the KAYAK (KAY) protein was shown to be involved in both the *Drosophila* molecular feedback loops underlying circadian oscillations (Ling et al., 2012). Finally, a very recent paper describes a role for CG17282/Bride of Doubletime (BDBT) in modulating DBT functions on PER (Fan et al., 2013). These evidences strongly support the effectiveness of the screen.

b. The Usp5 protein controls PER protein degradation

The *CG12082* gene encodes for a new protein involved in the regulation of the clock ticking. This polypeptide shows high sequence and structural similarities with the human Ubiquitin-specific peptidase 5 (Usp5)/Isopeptidase T (IsoT) protein, and we thus re-named the *Drosophila* gene *usp5*.

Downregulation of USP5 activities (*usp5*-RNAi expression) in clock neurons lengthened the circadian behaviors of flies and delayed PER and TIM oscillations in those cells. In addition, USP5 downregulation affected the size of the s-LN_v neurons, whose cell bodies (nuclei, in particular) appeared larger than those of wild type flies. Therefore, broad *usp5*-RNAi expression affected development of (at least) the s-LN_vs neurons. Interestingly, manipulation of the CLK and CYC levels (*cyc*⁰ and *Clk*^{Jrk} strongly hypomorph mutants) also affected growth and projections pattern of PDF⁺ clock neurons (Michard-Vahnee et al., in preparation) and led to arrhythmic behavior at the adult stage. Consequently, USP5 functions in LN_v development may in part underlie the effects of *usp5*-RNAi on the rhythmic behavior of adult flies.

Western blot analysis of RNAi-expressing fly head extracts showed higher CLK, PER and TIM protein quantities compared to controls. The PER protein was stabilized after RNAi induction also in a *tim* null background (*tim*⁰), while TIM did not accumulate in the absence of PER, suggesting that PER was the primary target of USP5 activities in the fruit fly clock machinery. Similar stabilizing effects of *usp5*-RNAi expression were shown also for CLK, indicating that USP5 controlled CLK stability independently of TIM but requiring PER. As a whole, data indicated that the PER protein is the main clock protein target for USP5 circadian functions, while the effects of *usp5* downregulation on the TIM and CLK protein are secondary.

The higher protein quantities shown in *usp5*-RNAi extracts was not a consequence of higher transcriptional rates. Indeed, the amplitude of the *Clk*, *per* and *tim* mRNA oscillations in *usp5*-RNAi flies was dampened compared to controls. However, the weaker *per* and *tim* transcriptional rates conflicted with the stabilization, in *usp5*-RNAi flies, of hypo-phosphorylated CLK at the end of the day, which should have prompted for higher transcriptional activities. The persistence of higher-phosphorylated PER repressor at those times likely limited CLK transactivation.

After RNAi induction, low-migrating PER and TIM proteins accumulated in head extracts at the end of the cycle (in the morning), while they underwent proteasomal breakdown in

wild-type flies at those times. The persistence of high molecular weight (phosphorylated) forms further indicated a default in the protein degradation processes. Interestingly, the protein encoded by the *CG12082* gene contains a N-terminal zinc-finger binding site and a C-terminal ubiquitin protease domain, and is highly related to human Ubiquitin-specific peptidase 5 (Usp5). Deubiquitylating enzymes (DUBs) regulate cellular ubiquitin amounts by reversing ubiquitin ligase activities. By undoing ubiquitylation, ubiquitin peptidases regulate, among other pathways, the proteasome-mediated protein degradation. Therefore, molecular data support a role for *CG12082/USP5* as an ubiquitin protease. However, additional evidences are required to prove that the high molecular weight PER and TIM proteins accumulating in the morning are effectively ubiquitylated.

c. Mechanisms for Usp5 control of clock protein degradation

Interestingly, among ubiquitin protease enzymes, human Usp5/IsoT was shown to play a more peculiar function than de-ubiquitylating ubiquitylated proteins. Usp5/IsoT specifically disassembles poly-ubiquitin chains to release free ubiquitin monomers, and restores the pool of cellular ubiquitin. In agreement, the *Drosophila* *CG12082/USP5* was strongly pulled-down in a tagged-Ub immunoprecipitation assays from wild-type larval neuronal extracts, indicating that *USP5* was an ubiquitin-conjugated protein (Franco et al., 2011). Unanchored poly-ubiquitin chains were shown to be toxic for the cell as they can compete with ubiquitylated proteins for proteasome binding and thus block proteasome-mediated protein degradation (Amerik et al., 1997; Dayal et al., 2009). However, multiple, substrate-specific mechanisms mediate proteasome recognition of ubiquitylated protein (Ciechanover and Stanhill, 2013). For instance, poly-ubiquitin chains may be created through polymerization at the internal Lys63 (K63 poly-ubiquitin chains) or using Lys48 (K48 poly-ubiquitin chains), the latter being a stronger signal for proteasome-mediated proteolysis. Moreover, ubiquitin receptors at the proteasome or specific ubiquitin-binding proteins may selectively control degradation of a subset of protein but not others. These pathways may be differently sensitive to accumulation of poly-ubiquitin chains (Dayal et al., 2009; Zhang et al., 2011). Indeed, Usp5 knockdown in human cells allowed stabilization of ubiquitylated p53 but not ubiquitylated Mdm2 protein (Dayal et al., 2009). Similarly, *Drosophila* *USP5* may control the degradation of a defined subset of clock components (whose ubiquitin-conjugation pattern is analogous), including PER, and not others (for example, TIM).

Recent studies have highlighted a number of physiological roles for poly-ubiquitin chains in the cell. Ubiquitin polymers can be pre-assembled at the level of E2 ubiquitin-conjugating enzymes and then transferred *in toto* to substrates (Li et al., 2007), which accelerates protein targeting. Interestingly, unanchored poly-ubiquitin chains were shown to directly promote auto-activation of the TAK1 protein kinase (Xia et al., 2009). Indeed, through regulating poly-ubiquitin chain accumulation, USP5 may affect the circadian molecular program in a number of different manners.

Ubiquitin-directed, proteasome-mediated clock protein proteolysis is a key step in the regulation of the pace of the clock. Indeed, a number of ubiquitin ligases that target PER, TIM and CLK for proteasome degradation have been identified (Grima et al., 2002; Ko et al., 2002; Lamaze et al., 2011; Grima et al., 2012). Yet, till date, only one ubiquitin-protease has been described, which circadian functions are unrelated to protein proteolysis. Indeed, the de-ubiquitylating activity of Ubiquitin-specific protease 8 (USP8) on CLK reduces transcription of CLK target genes and affects CLK molecular cycles but not CLK stability. Data presented here have indicated a circadian role for a second ubiquitin-protease, USP5.

Usp5 participates in the control of PER (and, secondarily, TIM) protein degradation during the daytime. We cannot exclude a “classical” role for USP5 in directly reversing PER ubiquitylation. However we are inclined to think that USP5 primarily control disassembly of poly-ubiquitin chains which, when accumulating (RNAi-mediated *usp5* downregulation), selectively block “PER-specific” protein degradation.

Cultured *Drosophila* S2 cells would be a better and convenient tool to test biochemical effects of USP5 knockdown or inhibition (Valinin A recently appeared as a USP5-specific inhibitor (Okada et al., 2013)). We are also collaborating with a group studying USP5/Leon effects at the synapse bouton, which will provide additional genetic tools to study the effect of USP5 overexpression or *usp5* mutations in adult flies.

II. THE STRIP-CONTAINING PP2A/CKA COMPLEX WORKS WITHIN THE *Drosophila melanogaster* CIRCADIAN CLOCK TO CONTROL DE-PHOSPHORYLATION OF THE CLOCK PROTEIN

Among the positive genes identified in our screen, great interest was dedicated to *CG11526*. *Drosophila* CG11526 protein, hereafter referred as STRIP, as well as its homolog in different species, has been found as part of a protein phosphatase 2A (PP2A)-containing super-complex, called STRIPAK (STRiatin Interacting Phosphatase And Kinase complex), which is well conserved across species.

The PP2A holoenzyme consists in a dimeric core of a catalytic (C) and a scaffold (A) subunits, and a variable regulatory (B) subunit. Multiple regulatory subunits exist that bind to the PP2A core enzyme in a mutually exclusive manner and determine the substrate specificity and localization of the PP2A holoenzyme. This illustrates the crucial role of the regulatory subunit for PP2A functions. Within the fruit fly STRIPAK complex, PP2A associates with CONNECTOR OF KINASE TO AP-1 (CKA), a novel PP2A regulatory subunit (Ribeiro et al., 2010).

In the fruit fly molecular clock, PP2A was already proposed to control the stability and nuclear entry of the PERIOD (PER) protein. These PP2A functions on PER were mediated by the WIDERBORST (WDB) or TWINS (TWS) regulatory subunits (Sathyanarayanan et al., 2004). In this work, we give evidences for the CKA-containing PP2A in the regulation of the *Drosophila* CLOCK (CLK) protein.

Downregulation of either *strip* or *Cka* favors the hyper-phosphorylation of CLK, the major transcriptional activator of the *Drosophila* molecular clockwork. The hyper-phosphorylated state of CLK is associated with transcriptional inhibition. Indeed, transcription of CLK target genes is reduced in *Cka*- or *strip*-RNAi expressing flies. Data are consistent with PP2A/CKA/STRIP complexes regulating CLK dephosphorylation to promote transcription of CLK-controlled genes.

In addition, we comparatively study the role of another known PP2A complex, containing the WDB regulatory subunit. We demonstrate that the two PP2A holoenzymes differently affect the CLK protein: CKA-containing PP2A adjusts cycle-specific CLK phosphorylation while PP2A/WDB complexes control CLK stability all over a daily cycle. Importantly, deregulation of total PP2A activities recapitulates the CLK hyper-phosphorylation caused by

CKA downregulation, strongly suggesting that CKA mediates the majority of PP2A function(s) upon CLK.

Finally, downregulation of STRIP, but not CKA, affects PER stability, indicating that STRIP possesses some CKA-independent circadian functions.

1. Article:

**The PP2A phosphatase complexes containing CKA (STRIPAK) or
WDB control different steps of CLOCK cycling in Drosophila**

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Running head: PP2A controls CLK oscillations

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Abstract

Circadian clocks rely on feedback loops where a transcriptional activating complex drives the expression of repressors of its activity. The pace of the oscillator largely depends on post-translational mechanisms that affect the stability and activity of the positive and negative components of the loop. In *Drosophila*, the CLOCK (CLK)/CYCLE (CYC) complex activates the expression of the *period* (*per*) and *timeless* (*tim*) genes in the evening, PER and TIM proteins then repress CLK activity in the late night. The CLK protein undergoes an oscillation of its phosphorylation with highly phosphorylated and inactive forms present in the morning and hypo-phosphorylated active forms present in the evening. How this critical dephosphorylation step is mediated is unknown. We show here that two components of the STRIPAK complex, the CKA regulatory subunit of the PP2A phosphatase and its interacting protein STRIP promote CLK dephosphorylation during daytime. In contrast, the WDB regulatory PP2A subunit stabilizes CLK but does not affect the apparent phosphorylation of the protein. Finally, we show that inhibition of the catalytic PP2A subunit affects CLK similarly to CKA downregulation, suggesting that CKA-promoted CLK dephosphorylation precedes WDB action on CLK.

Introduction

Circadian clocks govern a wide range of physiological and behavioral rhythms to adapt organisms to the 24h day-night cycles generated by the rotation of the earth on its axis. They stem upon feedback loops in which transcription factors activate the expression of their own inhibitors, which feedback with a timely regulated delay (Bell-Pedersen et al., 2005). In the *Drosophila* circadian oscillator, the CLOCK (CLK) and CYCLE (CYC) bHLH-PAS domain transcription factors activate expression of the *period* (*per*) and *timeless* (*tim*) genes at the end of the day (Hardin, 2011; Weber et al., 2011; Ozkaya and Rosato, 2012). In a second loop, CLK/CYC activate the *vrille* (*vri*) and *pdp1ε* genes, whose protein products repress (VRI) or activate (PDP1ε) the transcription of the *Clk* gene. The delayed accumulation of PER and TIM and their transfer to the nucleus leads to transcriptional repression of CLK/CYC during the late night. Subsequent degradation of PER and TIM repressors in the morning allows transcription to resume in the second part of the day. Controlled phosphorylation, ubiquitylation and proteasome-dependent degradation of PER and TIM set the timing of their delayed accumulation and clearance. The PER protein is phosphorylated by the DOUBLETIME (DBT, CK1δ/ε), CK2 and NEMO kinases and polyubiquitylated by the SCF^{Slimb} ubiquitin ligase complex (Kloss et al., 1998; Price et al., 1998; Grima et al., 2002; Ko et al., 2002; Lin et al., 2002; Akten et al., 2003; Nawathean and Rosbash, 2004; Chiu et al., 2008; Chiu et al., 2011). TIM associates with PER preventing its degradation, but TIM itself is subjected to phosphorylation and subsequent breakdown. TIM phosphorylation involves the CK2 and SHAGGY (SGG, GSK-3) kinases and TIM degradation also depends on SCF^{Slimb} and a Cullin-3-based ubiquitin ligase complex (Martinek et al., 2001; Grima et al., 2002; Lin et al., 2002; Meissner et al., 2008; Grima et al., 2012). PER and TIM phosphorylation is also controlled by phosphatase activity: PP2A regulates PER abundance while PP1 targets both PER and TIM (Sathyanarayanan et al., 2004; Fang et al., 2007).

CLK transcriptional activity depends on the phosphorylation status of the protein, which peaks in the morning and is at minimum in the early night (Lee et al., 1998; Kim and Edery, 2006; Yu et al., 2006; Hung et al., 2009). Similarly, CLK immunoreactivity in head extracts or brain tissue seems to oscillate in phase with its phosphorylation (Hung et al., 2009; Menet et al., 2010; Lamaze et al., 2011), although harsh extraction liberates chromatin-bound CLK which results in relatively constant CLK levels (Yu et al., 2006; Sun et al., 2010; Luo et al., 2012). Due to the cyclic regulation of CLK as opposed to constitutive expression of CYC, the CLK protein appears to represent the key rhythmic component of the circadian activator in

Drosophila (Bae et al., 2000). CLK DNA-binding and transcriptional activity show a robust oscillation with an evening peak that is associated with the rapid increase of *per* and *tim* mRNA levels (Yu et al., 2006; Menet et al., 2010; Abruzzi et al., 2011). The release of CLK from DNA correlates with its hyper-phosphorylation, which depends on both PER and DBT (Kim and Edery, 2006; Yu et al., 2006; Menet et al., 2010). Since kinase activity of DBT does not seem to be required for hyper-phosphorylation, it was proposed that DBT acts as an interface for the recruitment of other kinases into a complex with CLK (Yu et al., 2009). Kinases controlling CLK phosphorylation also include NEMO (NMO), which destabilizes CLK (Yu et al., 2011), and CK2alpha that stabilizes CLK and inhibits its transcriptional activity (Szabo et al., 2013). Other factors regulating CLK are the HECT-domain ubiquitin ligase CTRIP that destabilizes CLK (Lamaze et al., 2011), and the USP8 ubiquitin protease, which decreases its transcriptional activity (Luo et al., 2012). CLK activity is also shaped by other repressors/activators such as CLOCKWORK ORANGE (CWO) and the FOS ortholog KAYAK (KAY) (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008; Ling et al., 2012).

Phosphatases control the phosphorylation of clock proteins in various organisms and PP2A is the main source of serine/threonine phosphatase activity (Janssens and Goris, 2001; Virshup and Shenolikar, 2009). PP2A holoenzymes are predominantly heterotrimers in which a scaffold A and a catalytic C subunits associate with different types of regulatory B subunits, which define substrate specificity (Janssens and Goris, 2001; Shi, 2009; Virshup and Shenolikar, 2009). In *Drosophila*, the scaffold and catalytic subunits are encoded by the *pp2a-29b* and *microtubule star (mts)* genes respectively. Regulatory subunits are encoded by *twins (tws)* (B type), *widerborst (wdb)* and *PP2A-B'* (B' type), and *CG4733* (B'' type). The *Connector of kinase to AP-1 (Cka)* gene encodes a striatin that defines a B''' regulatory subunit (Ribeiro et al., 2010). B'''-containing PP2A holoenzymes have been found to be associated with striatin-interacting proteins and kinases in STRIPAK (STRiatin-Interacting Phosphatase And Kinase) complexes (Glatter et al., 2009; Goudreault et al., 2009; Ribeiro et al., 2010; Kean et al., 2011; Herzog et al., 2012). In *Drosophila*, the STRIPAK complex acts as a negative regulator of the HIPPO pathway (Ribeiro et al., 2010). CKA is required for the dorsal closure during embryonic development where it complexes with KAY and JUN-RELATED ANTIGEN (JRA) transcription factors to activate the expression of the *decapentaplegic (dpp)* gene (Chen et al., 2002). Whether this CKA function is mediated by the STRIPAK complex is not known.

PP2A activity is required for circadian rhythmicity in *Drosophila*. Loss of function mutations in the genes encoding the PP2A subunits are lethal, but overexpression of either a wild-type or a dominant negative form of the catalytic subunit induces behavioral arrhythmicity (Sathyanarayanan et al., 2004). In larval clock neurons, overexpression of wild-type MTS leads to constitutive high levels of PER, whereas expression of a dominant-negative MTS form leads to very low PER, supporting a PER stabilization function for PP2A activity (Sathyanarayanan et al., 2004). *wdb* and *tws* transcripts show circadian oscillations of their levels, and overexpression of the regulatory subunits lengthens (*wdb*) or slightly shortens (*tws*) the behavioral period (Sathyanarayanan et al., 2004). Downregulation of one or the other regulatory subunit induces PER degradation in S2 cells, suggesting that they associate with MTS to control PER oscillations (Sathyanarayanan et al., 2004). *wdb* or *tws* downregulation in S2 cells also induces CLK degradation (Kim and Edery, 2006), suggesting a CLK stabilization function for PP2A *in vivo*. The *Cka* gene has been identified as a CLK transcriptional target in the head (Abruzzi et al., 2011) and *Cka* mRNA levels cycle in intestinal cells (Karpowicz et al., 2013). Whether CKA-containing PP2A complexes contribute to clock mechanisms is not known. In this study, we investigated the role of the STRIPAK complexes in the control of the *Drosophila* clock. We show that STRIPAK complexes promote the dephosphorylation of the CLK protein specifically during daytime whereas PP2A complexes using the WDB regulatory subunit control CLK proteins levels around the clock.

Results

A large scale RNAi screen identifies STRIP as a new clock component

The NIGFly collection of RNAi lines was used to individually downregulate about 6000 genes using the GAL4/UAS expression system (Bouleau et al., unpublished). We used the *gal1118* GAL4 driver (Blanchardon et al., 2001), which mostly targets the clock neurons expressing the pigment-dispersing factor (PDF) neuropeptide. The locomotor activity of *gal1118>RNAi* flies was monitored in constant darkness (DD), and flies with altered behavioral rhythms were selected. We identified the *CG11526* gene, which encodes the *Drosophila* ortholog of the mammalian striatin-interacting protein (STRIP) (Goudreault et al., 2009; Ribeiro et al., 2010) and was thus named *strip*. *gal1118>strip-R1* RNAi flies showed a 2h lengthening of the behavioral period (Table 1 and Sup. Table 1). The target specificity of

the *strip-R1* RNAi was addressed by testing two additional non-overlapping *strip* RNAi sequences, which also induced long period rhythms when expressed in the clock neurons (Table 1, Fig. 1A and Sup. Fig 1). Long period phenotypes were also observed when *strip* RNAi and *dicer* were co-expressed under the control of the *tim-gal4* driver that is expressed in all clock cells (Table 1 and Fig. 1A). *tim>strip-R1* RNAi flies were tested for *strip* mRNA levels which showed a 70% decrease, indicating that the RNAi indeed targeted the *strip* transcripts (Sup. Fig 1). In addition, this experiment indicated that *strip* mRNA levels do not cycle in head extracts. PDF-expressing small lateral neurons (s-LNvs) were not morphologically altered in the RNAi flies (Sup. Fig 1) and PER immunofluorescence analysis revealed a delayed oscillation (Fig. 1B), in agreement with the long period of the behavioral rhythms.

A P-element insertion that is localized in the first intron of *strip* (Sup. Fig 1) was mobilized to generate deletions of the gene by imprecise excision (see Methods). A 4Kb deletion encompassing most of the *strip* gene was obtained but $\Delta 22130$ homozygous mutants died at the pupal stage. The PDF-expressing neurons of $\Delta 22130$ L3 larvae did not show obvious morphological defects (Sup. Fig 1) and were used for analyzing PER protein oscillations. PER levels were observed to cycle with a slow pace compared to heterozygous larvae, with a 6h delay at DD4 (Fig. 1B). This indicates that *strip* is not essential for rhythm generation but is required for having a 24h period.

***Cka* downregulation affects behavioral rhythms**

Since STRIP is found in the STRIPAK complex (Goudreault et al., 2009; Ribeiro et al., 2010; Kean et al., 2011), we asked whether STRIP function in the *Drosophila* clock would reflect a role for the STRIPAK complex. We thus asked whether the key component of the STRIPAK complex, the PP2A regulatory subunit CKA, has a circadian function. Expression of two non-overlapping *Cka* RNAi sequences in clock cells induced long period rhythms, indicating that CKA was indeed involved in the behavioral clock (Table 1, Fig. 2A and Sup. Fig. 2). Accordingly, *tim>Cka-R1* RNAi flies showed a 40% decrease of *Cka* mRNA levels (Sup. Fig. 2). No cycling of *Cka* transcripts nor of CKA protein was observed in head extracts (Sup. Fig. 2). The PDF cells were not altered in the RNAi flies (Sup. Fig. 2) and PER cycling was analyzed during the first two days of DD. Only a marginal delay of PER oscillation could be detected during the first two DD days (Fig. 2), and we thus decided to generate a more strongly affected genotype. *Cka*² null mutants are embryonic lethal (Chen et al., 2002) but viable adult flies could be obtained by providing daily heat-shock-controlled *Cka* expression

until the emergence of the adults. Developmentally rescued *Cka*² adults were then transferred to low temperature and did not show detectable CKA immunostaining in head extracts (Sup. Fig. 2). Flies were behaviorally tested at low temperature and became arrhythmic after a few days (Table 1 and Fig. 2). PDF-positive s-LNvs were normal in the mutants, but PER oscillations were abolished with low immunostaining levels at DD6 (Fig. 2). Our data indicate that both CKA and STRIP are involved in the circadian transcriptional feedback loop, strongly supporting a role for STRIPAK PP2A complexes.

STRIP and CKA prevent the accumulation of hyper-phosphorylated CLK during daytime

A Western blot analysis of head clock proteins was done to characterize the molecular defects associated with *strip* and *Cka* downregulation. *tim>strip-R1* flies showed delayed PER and TIM oscillations with hyper-phosphorylated forms observed at CT6-9 whereas such forms were almost absent at the same time in the controls (Fig. 3A). In agreement with a delayed molecular oscillation, newly synthesized low molecular weight PER and TIM were less abundant in the evening (CT9-12). *tim>Cka-R1* flies, which displayed a milder behavioral phenotype, had virtually no defect in PER/TIM cycling, at least in the first day of DD (Fig. 3B). However, developmentally rescued *Cka*² mutants showed a slightly delayed PER accumulation during the night, with less phosphorylated PER at CT12-18 (Fig. 3C). A different phenotype was observed for the CLK protein. During daytime, *tim>strip-R1* flies showed hyper-phosphorylated CLK forms with slightly decreased levels, and limited effects during the night (Fig. 3A). Hyper-phosphorylated CLK was also observed for *tim>Cka-R1* and *Cka*² flies during daytime (Fig. 3B). Flies expressing both *strip* and *Cka* RNAi molecules were generated and did not show a stronger phenotype compared to *strip* RNAi flies at both the behavioral and molecular levels (Table 1 and Sup. Fig 3). STRIP and CKA are part of the same STRIPAK complex in cultured *Drosophila* Kc167 cells (Ribeiro et al., 2010), but whether such complexes exist in fly heads is not known. STRIP and CKA also co-immunoprecipitated in *Drosophila* S2 cells (Sup. Fig 4). Then, flies carrying a tagged version of STRIP were generated and used for co-immunoprecipitation experiments with head extracts. STRIP and CKA did co-immunoprecipitate, indicating that the two proteins are part of the same complex in the adult head (Fig. 3D). Conversely, no STRIP-WDB nor STRIP-TWS complexes could be detected in head extracts.

Our results thus support a role for STRIPAK complexes containing STRIP and the CKA regulatory subunit of PP2A in CLK dephosphorylation during daytime.

STRIP and CKA require PER and DBT kinase activity to affect CLK phosphorylation

PER is required for CLK hyper-phosphorylation (Yu et al., 2006), and we thus asked whether STRIP and/or CKA would require PER to control CLK phosphorylation. Since *Clk* is expressed at very low levels in *per*⁰ mutants, we used the ARK transgenic flies that express *Clk* under the control of *per* regulatory sequences and thus have rather high CLK levels in the absence of PER (Kim et al., 2002). In a *per*⁰ ARK background, expression of either *strip* or *Cka* RNAi did not affect CLK phosphorylation, indicating that the phosphorylation events regulated by STRIPAK complexes are PER-dependent (Fig. 4A). CLK phosphorylation requires the DBT protein (Kim and Edery, 2006; Yu et al., 2006), although the presence of phosphorylated CLK in flies expressing a dominant negative form of the kinase (DBT^{K/R}) in a hypomorphic *dbt*^{AR} background supports the idea that DBT kinase activity is not required for CLK phosphorylation (Yu et al., 2009). We used the behaviorally arrhythmic *dbt*^{K/R}/*dbt*^{AR} flies to test the effects of *strip* and *Cka* downregulation on CLK. *strip* or *Cka* RNAi expression did not enhance CLK phosphorylation in a background with low DBT kinase activity, suggesting that DBT kinase activity is required for the phosphorylation events regulated by STRIPAK (Fig. 4B).

The persistence of daytime hyper-phosphorylated CLK in *Cka* or *strip* RNAi flies suggested that STRIPAK complexes might dephosphorylate CLK directly. We thus asked whether CLK associates with STRIP or CKA. Co-immunoprecipitation assays between CLK and either STRIP or CKA did not show the presence of CLK/STRIP or CLK/CKA complexes in head extracts (not shown). Since this interaction might be very labile and/or weak, we repeated these experiments in S2 cells where induction of high protein levels might compensate for weak interactions. CLK co-immunoprecipitated with both STRIP and CKA in the presence or in the absence of DBT (Fig. 4C), supporting the possibility that STRIPAK complexes directly dephosphorylate CLK.

CKA and WDB PP2A regulatory subunits have different effects on CLK

Downregulation of WDB and TWS PP2A regulatory subunits in S2 cells destabilizes PER (Sathyanarayanan et al., 2004), but also CLK (Kim and Edery, 2006). However, their function in adult flies has not been tested. We thus asked whether *wdb* or *tw*s downregulation would affect the behavioral clock. Although genotypes supporting a circadian function for *tw*s could not be obtained, expressing a *wdb* RNAi in the clock neurons induced behavioral defects. *tim*>*wdb-R1* flies had a 1h lengthening of the period in DD (Table 1 and Fig. 5A) and apparently normal PDF-expressing neurons (Sup. Fig. 5). Surprisingly, their mild behavioral

phenotype was associated to about 50% decreased *wdb* mRNA levels in head extracts, suggesting that *wdb* has a minor role than *Cka* in the behavioral clock. In agreement with their mild behavioral phenotype, *tim>wdb-R1* flies showed robust oscillations of the PER, TIM and CLK proteins with significantly lower levels of the three proteins, especially CLK (Fig. 5B). As for STRIP and CKA, no CLK-WDB complexes could be detected by co-immunoprecipitations in head extracts (not shown). Combining the expression of *wdb* RNAi with either *Cka* or *strip* RNAi produced additive effects on CLK with both decreased levels and hyper-phosphorylation during daytime (Fig. 5C). This suggests that WDB-PP2A on one hand and CKA-PP2A on the other hand have independent roles in the control of the CLK protein. We finally compared the effects of downregulating WDB and CKA regulatory subunits on CLK in S2 cells. Adding *wdb* dsRNA decreased CLK levels as previously reported (Kim and Edery, 2006), whereas *Cka* dsRNA induced a shift in CLK protein mobility (Sup. Fig. 5), likely reflecting CLK phosphorylation, in agreement with the head extracts results.

***mts* downregulation affects CLK similarly to *strip* or *Cka* downregulation**

Since WDB and CKA regulatory subunits have different effects on CLK, we asked how the inhibition of the PP2A catalytic subunit MTS would affect CLK. As previously reported (Sathyanarayanan et al., 2004), flies overexpressing a dominant negative form of MTS were arrhythmic in DD (Table 1 and Sup. Fig. 6). However, anti-PDF staining revealed that the PDF-neurons of *tim>mst^{DN}* flies were either missing or had strong morphological defects, preventing a molecular clock interpretation of the behavioral data (Sup. Fig. 6). Western blot analysis of *tim>mst^{DN}* head extracts showed the presence of hyper-phosphorylated PER and TIM during daytime (Fig. 6), in contrast to the decreased protein levels observed in *tim>wdb-R1* extracts. Hyper-phosphorylated CLK protein was also persisting during daytime, very similarly to what we observed in flies with downregulated *Cka* or *strip*. The results thus suggest that CKA-PP2A (STRIPAK) complexes are the predominant PP2A complexes for regulating CLK, and do so by promoting CLK dephosphorylation during daytime.

PP2A complexes increase CLK transcriptional activity

The CLK protein is transcriptionally active in the evening when low phosphorylated forms are present. Our data showed that *strip* and *Cka* downregulation strongly affected CLK phosphorylation, and we thus asked how they would affect CLK transcriptional activity. As a readout, we measured the levels of *tim* and *vri* pre-mRNAs, which show high amplitude

oscillations in wild-type flies (Fig. 7). In *tim>Cka-R1* and *tim>strip-R1* flies, *tim* and *vri* pre-mRNAs were significantly decreased at DD2. In contrast, *Clk* mRNA levels were not affected indicating that low CLK-dependent transcription was not a consequence of low *Clk* transcripts. Since deficient daytime CLK dephosphorylation occurs during daytime in the two genotypes (Fig. 3), it seems likely that it would prevent high efficiency CLK-dependent transcription. A decrease in pre-mRNA levels was also observed with pre-*tim*, but not pre-*vri*, in *tim>wdb-R1* flies. This genotype did not show CLK phosphorylation defects but had low CLK levels around the clock (Fig. 5). *Clk* mRNA levels were also decreased at all time points by about 40%, and this decrease likely participates to the low CLK levels. Since *wdb* downregulation but not *Cka* downregulation slightly increases PER phosphorylation, the transcriptional inhibition effects observed in *wdb* downregulated flies are likely a consequence of both constitutively low CLK and hyper-phosphorylated PER.

Discussion

The CLK/CYC complex is the activating factor in the negative feedback loop of the *Drosophila* clock and CLK is the regulated component of the complex. CLK is submitted to a phosphorylation cycle where CLK phosphorylation increases during the night to reach a peak in the morning. CLK is progressively dephosphorylated during the day and low phosphorylated CLK induces the transcription of its targets in the evening. The control of CLK phosphorylation cycle is poorly understood. Three kinases have been identified that affect CLK apparent molecular weight and/or stability. In addition, one study has reported that WDB and TWS PP2A regulatory subunits affect CLK stability in S2 cells. Our results identify two components of the STRIPAK complex, including the PP2A regulatory subunit CKA, as regulators of CLK phosphorylation. Whereas PP2A complexes containing the WDB regulatory subunit stabilize CLK in a time-independent fashion, CKA-containing STRIPAK complexes push hyper-phosphorylated CLK in the morning toward its low phosphorylated and transcriptionally active form in the evening.

The very similar CLK phenotype observed in *strip* and *Cka* RNAi flies suggests that the two genes work together. The finding of the two proteins in the same complex in adult head extracts strongly supports this idea. Since the two proteins belong to the STRIPAK complex (Ribeiro et al., 2010), it is very likely that STRIPAK promotes CLK dephosphorylation. A PER/TIM phenotype was observed in *strip* but not in *Cka* RNAi flies. No hyper-

phosphorylation could be detected but rather a slowed down oscillation, likely reflecting the longer behavioral period of the *strip* RNAi flies. However, a *strip*-specific function cannot be excluded that could be either unrelated to PP2A or be a consequence of other STRIP-containing PP2A complexes. STRIP-containing PP2A complexes with no CKA have not been reported in flies or mammals. However, the STRIP-like protein FAR11 was found in PP2A complexes containing a B'-type PP2A regulatory subunit in yeast (Pracheil et al., 2012).

STRIPAK complexes are still poorly understood, but they are involved in various cytoplasmic processes including Golgi positioning and cytokinesis, indicating a role in the cytoskeleton organization (Tully, 1987; Kean et al., 2011; Hyodo et al., 2012). In *Drosophila*, the STRIPAK complex has been shown to positively regulate tissue growth by promoting the dephosphorylation of HIPPO (HPO), which binds to both CKA and STRIP (Ribeiro et al., 2010).

Drosophila CLK has not been observed in the cytoplasm of the clock neurons and we could not detect CLK sub-cellular localization defects in *strip* or *Cka* downregulated flies (not shown). How the cytoplasmic STRIPAK complex might regulate CLK phosphorylation?

A PER-directed cytoplasmic transfer of presumably highly phosphorylated CLK has been reported in S2 cells (Kim and Edery, 2006). Other studies report the presence of low phosphorylated CLK in the cytoplasm of S2 cells, with CYC promoting its phosphorylation and the subsequent transfer of the hypo-phosphorylated active CLK in the nucleus (Hung et al., 2009; Maurer et al., 2009). It is thus possible that cytoplasmic CLK is present but not detected in the *Drosophila* clock neurons. In *Neurospora*, the WCC functional homolog of CLK is also transcriptionally active and unstable as a low phosphorylated form whereas its FRQ-promoted phosphorylation inhibits and stabilizes it (Yang et al., 2004; Schafmeier et al., 2008). Highly phosphorylated WCC is shuttled to the cytoplasm and then dephosphorylated by PP2A before re-entering the nucleus to resume transcription as an active and unstable form (Yang et al., 2004; Schafmeier et al., 2008). In mammals, the CYC ortholog BMAL1 is the regulated component of the transcriptional activator complex (Partch et al., 2013). CLK-BMAL1 complexes are found in the cytoplasm and BMAL1 nucleo-cytoplasmic shuttling appears to promote both the activity and the instability of the nuclear complex (Kondratov et al., 2003; Kwon et al., 2006). Another possibility is that STRIPAK complexes favor the daytime nuclear localization of a phosphatase activity. In *Neurospora*, STRIPAK complexes associate with the nuclear membrane and control the nuclear accumulation of a MAP kinase (Dettmann et al., 2013). However the finding of CKA/CLK and STRIP/CLK complexes support a CLK/STRIPAK co-localization. Finally, STRIPAK complexes could localize to the

nucleus. In cultured cells, transfected CKA is mostly cytoplasmic but partially relocalize to the nucleus when co-transfected with KAY and JRA transcription factors (Chen et al., 2002).

Interestingly, KAY has been suggested to buffer CLK/CYC activity by activating a CLK/CYC inhibitor on one hand, and inhibiting the *Clk* gene repressor VRI on the other hand (Ling et al., 2012). Since we have seen no change in *Clk* mRNA levels in *Cka* RNAi flies, CKA does not seem to control KAY-dependent VRI inhibition. However, the activation of KAY/JRA-dependent transcription by CKA (Chen et al., 2002) would predict an enhanced expression of the CLK targets in *Cka* downregulated flies, in contrast to what we have observed. Thus, CKA is either not involved or has an opposite effect on the KAY-dependent repression of CLK activity. Alternatively, CKA might have two opposite functions, with CLK dephosphorylation predominating on KAY activation. As for the STRIPAK-dependent dephosphorylation of HPO, it is not known whether CLK is directly dephosphorylated by the MTS PP2A catalytic subunit of the STRIPAK complex. The STRIPAK complex contains several kinases, whose PP2A-induced inhibition could decrease CLK phosphorylation. Members of the Ste-20 kinase family, such as *misshapen (msn)* and *germinal center kinase III (gck-III)*, co-purified with CKA in S2 cells (Ribeiro et al., 2010). It will be interesting to see whether such kinase components affect the molecular clockwork.

The medium-phosphorylated CLK profile shown by *dbt^{K/R}*, *dbt^{ar}* flies was not altered by RNAi-mediated downregulation of either *strip* or of *Cka*, suggesting that *dbt* acts upstream of CKA-PP2A to regulate CLK. In contrast to DBT, inhibition of the CK2 α kinase (*CK2 α ^{Tik}* overexpression) did not prevent the CLK hyper-phosphorylation effects of *Cka* downregulation (not shown). Therefore, CK2 α and CKA-PP2A likely affect CLK in a completely independent manner. Since downregulation of WDB and TWS PP2A destabilize CLK in S2 cells, it has been proposed that CLK hyper-phosphorylation and degradation are controlled by a balance between PP2A and DBT (Kim and Edery, 2006). Our results in flies demonstrate that WDB strongly stabilizes CLK and thus play a very different role compared to CKA. However, we did not detect obvious changes in CLK protein mobility on gels and the stabilizing effects of WDB occur all around the clock, questioning the role of WDB in such a balance between DBT and WDB activities. TWS might be more involved in counteracting DBT-mediated CLK phosphorylation, but tools are lacking to inhibit TWS in adult flies. MTS downregulation affected CLK very similarly to CKA downregulation, with higher phosphorylated CLK during the day compared to wild type. In contrast, the low CLK levels of the *tim>wdb* RNAi flies were not observed in *tim>mts^{DN}* flies. Although a MTS-independent role of WDB cannot be excluded, it suggests that CKA-MTS activity is required

for WDB-MTS activity on CLK at least during daytime. In a wild type situation, CKA would bring highly phosphorylated CLK in a lower phosphorylation state that makes it a good target for stabilization by WDB. Since CLK is strongly destabilized by *tws* downregulation in S2 cells (Kim and Edery, 2006), the *mts^{DN}* results suggest that TWS also needs CKA activity to protect CLK from degradation at least during daytime.

Our qRT-PCR results indicated that downregulation of both WDB and CKA-PP2A complexes decreases CLK-dependent transcription. *Cka*-RNAi severely reduced *tim* pre-mRNA levels and also affect *vri*, whereas *wdb* downregulation strongly affected *tim* pre-mRNAs with no detectable impact on *vri*. This seems to reflect *tim>Cka-R1* flies having a longer behavioral period (25.9 h) than *tim>wdb-R1* flies (25.1 h). It could also suggest that *vri* circadian transcription might be less sensitive than *tim* transcription to CLK defects. More importantly, these effects need to be compared to the CLK molecular defects observed in the *Cka* or *wdb* downregulated flies. They indicate that CLK phosphorylation, rather than CLK levels, is a key parameter for CLK activity. Although higher CLK levels are associated with a shorter period (Kadener et al., 2008), significant differences in CLK levels have been reported to have limited effects on the pacemaker (Kim et al., 2002). Further support to this idea comes from *CK2 α ^{Tik}* flies, in which low levels of hypo-phosphorylated CLK display strong transcriptional activity (Szabo et al., 2013).

The regulation of CLK activity is a central point in the functioning of the circadian oscillator. Yet, it is a poorly understood aspect of the *Drosophila* clock. Our study shows that CLK activity is regulated by at least two types of PP2A complexes that work very differently. STRIPAK CKA-PP2A complexes play a key role in the dephosphorylation of the hyper-phosphorylated CLK that accumulates in the morning. In contrast, WDB-PP2A complexes target less phosphorylated CLK and thus require CKA-PP2A activity to control the CLK protein levels. It thus appears that STRIPAK complexes initiate the refueling of the pool of hypo-phosphorylated active CLK that will allow a novel cycle of transcription. It remains to be determined whether CLK recycling includes nucleo-cytoplasmic exchanges, such as those occurring for the WCC activating complex in *Neurospora*, or suggested for the mammalian CLK protein.

Experimental Procedures

Fly strains and constructs

Flies were maintained on standard cornmeal-yeast-agar medium on 12h:12h LD conditions. Transgenic flies carrying UAS-RNAi transgenes were obtained from the NIG-Fly (<http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp>), VDRC (<http://stockcenter.vdrc.at/control/main>) or TRiP (<http://www.flyrnai.org/TRiP-HOME.html>) collections, as indicated. *strip*-R1: NIG-FLY #11526-R1, *strip*-R2: VDRC #16211, *strip*-R3: VDRC #16212; *strip*-R4: VDRC #106184, *strip*-R5: TRiP #34657, *strip*-R6: NIG-FLY #11526-R2. *Cka*-R1: VDRC #35234, *Cka*-R2: VDRC #35232, *Cka*-R3: VDRC #106971, *Cka*-R4: TRiP #31642, *Cka*-R5: Trip #28927. *wdb*-R1: TRiP #28939. *Cka*² null mutants are embryonic lethal (Chen et al., 2002). To obtain adults, *Cka*² mutants bearing an *HS-Cka* transgene (*w*; *FRT40A Cka*² and *w*; *HS-Cka* flies were kind gift from S. X. Hou) were heat-shocked twice a day for 30 min until hatching. Adults were then kept at low temperature for at least 7 days before testing. *w*; *UAS-dbt*^{K/R} flies, that over-express a dominant negative dead version (K38R substitution) of the DBT kinase (Muskus et al., 2007) (line 12F1A), and *w*; *ARK8.2/CyO*; flies, bearing the *Clk* ORF under control of *per* regulatory elements (Kim et al., 2002) were kindly provided by J. Price and I. Edery, respectively.

w; *UAS-mts*^{DN} flies (Hannus et al., 2002) were generously provided by S. Eaton. The altered *mts* transgene we used possess 181 bp deletions at its N-terminal sequence. *Drosophila strip* ORF was PCR amplified from the AT25231 cDNA clone (Drosophila Genomic Resource Center, DGRC) and ligated into a Gateway (Invitrogen) compatible entry vector (pENTR-D-TOPO). The following primer pair was used for cDNA amplification: 5'-CACCATGGCTTGGAAACAGAAAAATAAAA-3' and 5'-GTTAATAACTTCTTGCTTTTATGACGC-3'. By LR recombination the sequenced ORF was transferred from the entry vector into a pTFW destination vector (Drosophila Gateway Vector collection). The clone obtained allowed for Gal4-driven somatic *in vivo* expression (UAS promoter) of the *strip* protein fused to a triple Flag tag at its N-terminus. This final construct was sequenced and then stably introduced into flies as a P element by germinal injection (FlyFacility). Transgenic flies were recognized by mini-white expression from the P element giving red eyes in an otherwise *w* background.

Deletion of the *strip* locus was generated by imprecise excision of the HP37079 P(EPg) transposable element (Bloomington #22130) using a $\Delta 2.3$ transposase source identified by the *Sb* marker. Several stable independent lines were established that had lost the *mini-w*

marker associated to the P element insertion and the *Sb* phenotype associated to the transposase source. Flies from homozygous viable jump out lines were tested for altered behavioral phenotypes, without success. Only one line failed to lose the *TM6,Tb* balancer chromosome and showed pupal lethality. The genomic DNA of homozygous mutant larvae was analyzed by PCR using multiple primer couples designed into *more and more* distant regions flanking the insertion site. We obtained a ≈ 5 Kb PCR amplification product using primers PR525: 5'-GTGAAGGAATTACTGGTGTGGATG-3' and PR578: 5'-CCTGTGGTGGCAAACTACGC-3', which was sequenced using internal primers PR595: 5'-ATCGCATCGTCCACTACTTCAAG-3' and PR596: 5'-AACTGGGCACTTTCTCACTTTTCG-3'. Sequencing reveals the complete loss of exons 2 to 4 of the *CG11526* gene span (deletions spans between 3L: 3322044, 3326128). Even if deletion does not include exons of the *PHGPx* gene (located in the *CG11526* first intron on opposite direction to *CG11526*), and only a non-coding region of the last *CG12016* exon (3' to *CG11526*, on opposite direction) we cannot exclude that perturbed PHGPx or CG12016 gene expression may be responsible of the lethality.

Behavioral analysis

Locomotor activities of individual flies were measured as previously described using *Drosophila* monitoring system from TriKinetics (Hamblen-Coyle et al., 1989). Briefly, young adult males were entrained to 12:12 LD (12h light followed by 12h dark) cycles at 25°C for 2 to 4 d, and subsequent kept in constant dark conditions (DD) for 8-11 d. The locomotor activity data of each individual fly was analyzed using the FaasX (Fly activity analysis suite for MacOSX) 1.16 software (Klarsfeld et al., 2003). Actograms are double-plotted graphs representing the absolute activity levels for each 0.5h interval, averaged over group of flies of a given phenotype. The hash density (HD= number of activity events per hash) varies from 12 to 35 according to the activity levels of the genotype. This allows the comparison of activity profiles between genotypes that display very different activity levels. Periods were calculated starting from the second day of constant darkness for each individual fly using a χ^2 periodogram analysis (CycleP) and pooled to calculate a group average for each independent transgenic line or genotype. The following filtering criteria to assess rhythmicity were used: power ≥ 20 , width ≥ 2 h. Power indicates the robustness of the rhythm in DD; width denotes the number of peaks in 30 min increments above the periodogram 95% confidence line. Genotypes with a reduced number of rhythmic flies (<50%), low power

(<50) and high s.e.m. of the period (>1) are considered arrhythmic. All behavioral experiments were reproduced at least two times with very similar results.

Western blotting

20 to 40 flies (both males and females) were collected on dry ice at indicated time points and processed as previously described (Lamaze et al., 2011; Szabo et al., 2013). Isolated frozen heads were homogenized in ice-cold RBS lysis buffer (10 mM HEPES at pH 7.5; 5 mM Tris-HCl at pH 7.5; 50 mM KCl; 2 mM EDTA at pH 8.0; 10% glycerol; 1% Triton X-100; 0,4% NP-40) supplemented with anti-proteases tablets (Roche), phosphatase inhibitor cocktail 2 and 3 (Sigma) and 20 mM β -glycerophosphate. To improve extraction efficiency, extraction was performed in two times. Tubes were homogenized for 1 min, followed by 10 to 15 min of ice incubation, and then homogenized again for another 15 to 30 seconds. Protein concentration was determined by using a Bradford (BioRad) protein assay, according to the manufacturer's instructions. For SDS-PAGE, 50 μ g of protein were separated on NuPAGE Novex 4% or 6% Tris-Glycine precast gels (PER, TIM and CLK immunoblotting) or 4-12% Bis-Tris gels (GFP and WDB immunoblotting) from Invitrogen by Life Technologies, and transferred to PVDF membranes. Primary antibodies were used at the following dilutions: goat anti-CLK sc-27070 (Santa Cruz Biotechnology), 1:1,000; rabbit anti-PER (Stanewsky et al., 1997), 1:10,000; rat anti-TIM (Grima et al., 2002), 1:4,000; rabbit anti-CKA (a kind gift from S.X. Hou) 1:2,000; guinea-pig anti-WDB (kindly provided by A. Sehgal) 1:5,000; rat anti-TWS (a gift from T. Uemura) 1:500. HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were used as follow: anti-goat donkey antibody 1:14,000; anti-rabbit goat antibody 1:40,000; anti-rat goat antibody 1:40,000. Chemiluminescence reaction was performed using Pierce ECL-Plus Western Blotting Substrate (Thermo Scientific). All of these immunoblot analyses were repeated in two or three experiments, most of which were blotted multiple times.

Immunoprecipitation

For immunoprecipitation assays frozen heads were homogenized in ice-cold HE lysis buffer (20 mM HEPES pH 7.5; 100 mM KCl; 10 mM EDTA pH 8.0; 0,1% NP-40; 5% glycerol) supplemented with anti-proteases tablets (Roche), phosphatase inhibitor cocktail 2 and 3 (Sigma) and 20 mM β -glycerophosphate. For FLAG immunoprecipitations, 600 μ g to 1 mg of total protein extract was incubated overnight at 4°C with 25 μ L EZview Red anti-FLAG M2 Affinity Gel (Sigma-Aldrich). Other immunoprecipitations were performed using 25 to 50 μ L

Protein G Sepharose 4 Fast Flow (GE Healthcare) pre-coupled with 10 μ L of goat anti-CLK antibody (sc-27069, Santa Cruz Biotechnology) or 3 μ L mouse anti-HA antibody (12CA5, Roche) or 10 μ L goat anti-c-MYC antibody (sc-789-G, Santa Cruz Biotechnology). Beads were washed three times for 45 min with HE lysis buffer. Purified protein and associated complexes were then eluted in 1X NuPAGE LDS Sample Buffer (Invitrogen by Life Technologies), 1X Sample Reducing Agent (Invitrogen by Life Technologies) for 10 min at 70°C. The same protocol was used for S2 cells immunoprecipitation analysis.

Cell culture experiments

Drosophila Schneider 2 (S2) cells (kind gift of Anne Plessis) were maintained in Schneider's *Drosophila* Medium (Gibco by Life Technologies) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin solution (Sigma-Aldrich). Complementary single-stranded RNAs were *in vitro* transcribed from purified PCR templates containing the T7 RNA polymerase promoter site on both ends, using the MEGAScript T7 Kit (Ambion by Life Technologies). Reactions were purified with the MEGA Clear Kit (Ambion by Life Technologies) and precipitated in ethanol/sodium acetate. dsRNAs were re-suspended in 50 μ L ultra-pure H₂O and the two strands re-annealed (30 min at 65°C and slow cooling to RT). RNA quality and quantity were assessed by spectrophotometry and agarose gel electrophoresis. Primers for the target sequence amplification by PCR are described below.

strip:

5'-taatacgaactcactatagggACTCCTTTGAGCAGGACACCC-3'

5'-taatacgaactcactatagggGTCCGTGGAGTCCACAAGCGT-3'

Cka:

5'-taatacgaactcactatagggATACGGGTCCAGTTCTGTGC-3'

5'-taatacgaactcactatagggTCGTCAAACCTTCTTGCGATG-3'

wdb:

5'-taatacgaactcactatagggGGAGGACGATCCGACACTGGA-3'

5'-taatacgaactcactatagggATGATCGGCATGATGACCGCA-3'

luc:

5'-gaattaatacgaactcactatagggagaGGCTATGAAGAGATACGCCC-3'

5'-gaattaatacgaactcactatagggagaTCCACATATCAAATATCCGA-3'

pAct-*Clk*-V5 was a gift from J. Menet; pAc-DBT-V5 was kindly provided by I. Edery. pMt-*Cka*-Flag-HA and pMt-*strip*-Flag-HA were created at BDGP (Berkeley *Drosophila* Genome

Strip: 5'-TCGATTGAAGGTGGGTTTGG-3' and 5'-GGGAGCGTTTGAAGTTCCG-3';
Cka: 5'-GGAGACGGAAGGCCGTCATA-3' and 5'-TCTCGTCGTCGTCGGACATC-3';
wdb: 5'-GTGGCCTGCTGAAGTTCTGG-3' and 5'-GCTCGCTCAGCAACCTGAAA-3';
Clk: 5'-ATCGCGTAATCTCAGCGAGAA-3' and 5'-CGATCGGTGGCCTCATTATG-3';
pre-tim: 5'-ACTGCTTTCCAATGCGGTATG-3' and 5'-AAGCCTCCGAAAAACATATGAAAA-3';
pre-vri: 5'-CAAGCAGGATAATCCCAGCAA-3' and 5'-ACAGATTTCAAGATCAAACGTGGA-3'.

Specificity and absence of multi-locus matching at the primer site were verified by BLAST analysis. The amplification efficiencies of primers were generated using the slopes of standard curves obtained by a ten-fold dilution series of 4, with all experimental points falling within this range. Amplification specificity for each q-PCR reaction was confirmed by analysis of the dissociation curves. Determined Ct values were then used for quantification, with the *tubulin* gene as reference. Each sample measurement was made at least in duplicate (technical replicate).

Brain immunostainings

Larval and adult flies were collected at the indicated circadian times (CT) and dissected. Samples were incubated 3 to 4 days with primary antibodies and overnight with secondary antibodies. Primary antibodies were: rabbit anti-PER (Stanewsky et al., 1997) 1:15,000; guinea pig GP-47 anti-CLK (Houl et al., 2006) 1:15,000; mouse anti-PDF (Developmental Studies Hybridoma bank) 1:50,000. Secondary antibodies were: anti-rabbit Alexa Fluor 647 1:5,000; anti-guinea pig Alexa Fluor 488 1:2,000; anti-mouse Alexa Fluor 594 1:2,000 (Invitrogen by Life Technologies). Whole brains were mounted in slides with ProLong (Roche) mounting medium and imaged using a Zeiss AxioImager Z1 fluorescence microscope equipped with AxioCam MRm digital camera (Zeiss) and an ApoTome structured illumination module (Zeiss). Fluorescence intensity of individual cells was quantified from digital images of single focal planes (Axio Vision program) with the NIH ImageJ software. To count for different exposition times (thus background intensity) we calculated a fluorescence index applying the formula: $I=100*(S-B)/B$ (S (Signal) is the fluorescence intensity of the positive cell; B (Background) is the average intensity of the region adjacent to the positive cell), which gives the fluorescence percentage above background. Index values were then averaged for the 4 PDF-positive s-LN_vs of 12 to 20 brain hemispheres for each time point.

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Figure Legends

Table 1: Locomotor activity rhythms in DD.

Flies were kept at 25°C and exposed to at least 2 days of 12:12 LD followed by 11 days of DD conditions. Rhythmicity (R) is calculated using the last 10 days of experiment. Results are referred to male flies; except for *Cka* rescued flies (*Cka*²,*HS-Cka*) for which only homozygous females emerged.

^a Total number of flies that survived until the end of the testing period

^b Percentage of flies with activity rhythms having a power value of ≥ 20

^c Measure of the strength or amplitude of the rhythm.

Figure 1: *strip* is required for normal behavioral and molecular rhythms.

A. Representative actogram records showing the average locomotor activity of *tim>strip-R1*, *tim>strip-R2* and control flies. *strip-R1* and *strip-R2* RNAi lines recognize two non-overlapping regions on the *strip* mRNA. One day of 12h:12h LD condition is followed by 9 days of constant darkness (DD) at 25°C. A shaded background indicates dark phase. On the top of the graph, black rectangles indicate night phases, while white and grey rectangles indicate day and subjective day phases, respectively. N: number of flies that survive until the end of the experiment. The hash density (HD) varies according to the genotype (see Experimental procedures). **B. Left** Oscillations of the PER protein levels in the s-LN_v neurons of UAS-*strip*-RNAi expressing flies (dotted line) compared to controls (full line), Flies were entrained in LD for 4 days and then placed in constant darkness. Ten flies were dissected every 3h during the last day of LD condition and the following first day of constant darkness. **Right** Oscillations of the PER protein levels in the s-LN_v neurons of *strip* homozygous mutant L2/L3 larvae (*Δ22130* - dotted line) compared to heterozygous siblings (full line). *Δ22130/TM6Tb* flies were left laying eggs in tubes under standard LD conditions for 4 days. Parental flies were then removed and tubes with eggs placed in constant darkness for 3 days before dissections on the 4th day of DD. Homozygous *Δ22130* larvae were distinguished from heterozygous siblings by their size (*Tb* phenotypic marker). Fluorescence index is given in arbitrary units. Error bars indicate s.e.m. Black rectangles indicate the night phase; white rectangles are for the day phase, while grey rectangles indicate subjective day phases.

Figure 2: *Cka* is required for normal behavioral and molecular rhythms.

A. Top Representative actogram records showing the average locomotor activity of *tim>Cka-R1* and *tim>Cka-R5* flies, compared to *tim>+* controls. *Cka-R1* and *Cka-R5* RNAi lines recognize two non-overlapping regions of the *Cka* mRNA. One day of 12h:12h LD condition is followed by 9 days of constant darkness (DD) at 25°C. **Bottom** Average actogram records of adult *Cka* mutant flies (*Cka*²) rescued for *Cka* during development (see Experimental Procedures) and tested at cold temperature (19°C). The graph represents one day of 12h:12h LD condition, followed by 9 days of DD. For all actograms, a shaded background indicates dark phase. On the top of the graph, black rectangles indicate night phases, while white and grey rectangles indicate day and subjective day phases, respectively. N: number of flies that survive until the end of the experiment. The hash density (HD) varies according to the genotype (see Experimental procedures). **B. Left** Oscillations of the PER protein levels in the s-LN_v neurons of *Cka*-RNAi expressing flies (dotted line) compared to controls (full line). Flies were entrained in LD for 4 days and then placed in constant darkness. Ten flies were dissected every 3h during the first two days of constant darkness (DD1-DD2). **Right** Oscillations of the PER protein levels in the s-LN_v neurons of homozygous *Cka* adult mutants (dotted line) compared to heterozygous siblings (full line). Flies were entrained in LD for 4 days, then placed in constant darkness and dissected every 3 hours at DD6. Fluorescence index is given in arbitrary units. Error bars indicate s.e.m. Black and grey rectangles indicate the subjective night and subjective day phases, respectively.

Figure 3: CKA and STRIP promote CLK dephosphorylation

Western blot of fly head extracts collected every 3h during the first day of constant conditions after 4 days of entrainment in 12h:12h LD at 25°C. For each time point, extracts from control flies are compared to extracts of RNAi-expressing or mutant flies. Genotypes are indicated. P-CLK: hyper-phosphorylated CLK. P-PER: hyper-phosphorylated PER. P-TIM: hyper-phosphorylated TIM. **A.** Comparison of CLK, PER and TIM proteins in *tim>strip*-RNAi and *tim>+* control flies. **B.** Comparison of CLK, PER and TIM proteins in *tim>Cka*-RNAi and *tim>+* control flies. **C.** Comparison of CLK, PER and TIM proteins in homozygous *Cka* mutants and heterozygous siblings (control). Black and grey rectangles indicate the subjective night and subjective day phases, respectively. Experiments were performed at least twice. **D.** A Flag(Fg)-tagged version of *strip* was broadly overexpressed in flies using the *tim-gal4* driver. *strip* overexpressing flies and relative controls were entrained in standard 12h:12h LD condition and collected every 6h during the following day of constant darkness. Heads were

lysed and extracts quantified. Two different quantities (a= 0,7 mg, b= 1,4 mg) of total protein extracts were used as input for an anti-Flag immunoprecipitation assay. Supernatant (containing proteins that had not bound anti-Flag-tagged beads) and immunocomplexes were recuperated and run into a gel. 50 µg of head extracts were loaded as an input control. Transferred membranes were incubated with anti-CKA. Black and grey rectangles indicate the subjective night and subjective day phases, respectively.

Figure 4: Effects of DBT and PER on STRIP and CKA function

A-B. Western blot of head extracts from flies collected at DD1 and DD2. Time (h) is indicated as CT. P-CLK: hyper-phosphorylated CLK. **A.** Comparison of CLK protein profiles in *tim>Cka*-RNAi (c) or *tim>strip*-RNAi (s) and control (C) flies in a *per*⁰ background. CLK overexpression from a *per* promoter (*ARK* flies) is used to increase CLK amounts. **B. Top:** overexpression of a dominant-negative kinase-dead allele of the Dbt kinase (*dbt*^{K/R}, *dbt*^{ar} = d) allows non-cycling phosphorylated CLK to appear. A heterozygous *dbt*^{ar} mutant background is used to dampen endogenous wild-type DBT activity. **Bottom** Comparison of CLK protein profiles in *tim>Cka*-RNAi (c) or *tim>strip*-RNAi (s) and control (C) flies in a *dbt*^{K/R},*dbt*^{ar} (d) mutant background. Genotypes are indicated. P-CLK: hyper-phosphorylated CLK. *dbt*^{K/R} and *dbt*^{ar} are *dbt* alleles already reported. **C.** *Drosophila* S2 cells were transfected with *pMt-strip-FgHA* (*dSp* - 400 ng) and *pMt-Cka-FgHA* (*Cka* - 400 ng) plasmids, and *pAct-Clk-V5* (*Clk* - 400 ng), *pAct-dbt-V5* (*Dbt* - 100 ng) or equal amounts of an empty vector, as indicated. Cell lysates were subjected to anti-CLK, anti-βgal and anti-myc immunoprecipitations (IPs). Input extracts, IP of CLK and co-IP of CKA and strip were visualized by immunoblotting. The position of the molecular weight markers is indicated on the right side.

Figure 5: Comparison between CKA and WDB activities.

A. Representative actogram records showing the average locomotor activity of *tim>wdb*-RNAi and control *tim>+* flies. One day of 12h:12h LD condition is followed by 9 days of constant darkness (DD) at 25°C. A shaded background indicates dark phase. On the top of the graph, black rectangles indicate night phases, while white and grey rectangles indicate day and subjective day phases, respectively. N: number of flies that survive until the end of the experiment. The hash density (HD) varies according to the genotype (see Experimental procedures). **B.** Western blot of head extracts of flies collected during the first day of constant conditions after 4 days of LD entrainment at 25°C. For each time point, extracts from control flies are compared to extracts of RNAi expressing flies. Black and grey rectangles

indicate the subjective night and subjective day phases, respectively. Time (h) is indicated as CT. P-CLK: hyper-phosphorylated CLK. P-PER: hyper-phosphorylated PER. P-TIM: hyper-phosphorylated TIM. **B.** Comparison of CLK, PER and TIM profile in *tim>wdb*-RNAi (w) and control *tim>+* (C) flies. **C.** Comparison of CLK profile in *tim>wdb*-RNAi (w), *tim>Cka*-RNAi (c), *tim>wdb*-RNAi, *Cka*-RNAi (cw) and control *tim>+* (C) flies.

Figure 6: Molecular analysis of *mts* function

Western blot of head extracts of flies collected every 3h during the first day of constant conditions after 4 days of entrainment in 12h:12h LD at 25°C. For each time point, extracts of control flies (*tim>+*, C) are compared to extracts of *tim>mts^{DN}* (M) flies. *mts^{DN}* is a dominant-negative allele for the PP2A catalytic subunit. Black and grey rectangles indicate the subjective night and subjective day phases, respectively. Time (h) is indicated as CT. P-CLK: hyper-phosphorylated CLK. P-PER: hyper-phosphorylated PER. P-TIM: hyper-phosphorylated TIM.

Figure 7: Transcriptional effects of *cka*, *strip* and *mts*

Quantitative RT-PCR measurements of *tim* (*pre-tim*, left column) and *vri* (*pre-vri*, middle column) pre-mRNA and *Clk* mRNA (right column) levels in head extracts of flies collected every 3h during DD2. Results shown are means of pooled values from at least three independent experiments. Error bar indicate s.e.m. Average values are normalized to the mean of peak control values set to 100. Black and grey rectangles indicate the subjective night and subjective day phases, respectively. Time (h) is indicated as CT. **Top** Comparison of extracts from *tim>strip*-RNAi and control *tim>+* flies. **Middle** Comparison of extracts from *tim>Cka*-RNAi and control *tim>+* flies. **Bottom** Comparison of extracts from *tim>wdb*-RNAi and control *tim>+* flies.

Supplemental Data

Supplemental Table 1: Locomotor activity rhythms in DD

Flies were kept at 25°C and exposed to at least 2 days of 12:12 LD followed by 11 days of DD conditions. Rhythmicity (R) is calculated using the last 10 days of experiment. Results are referred to male flies.

^aTotal number of flies that survived until the end of the testing period

^bPercentage of flies with activity rhythms having a power value of ≥ 20

^cMeasure of the strength or amplitude of the rhythm.

Supplemental Figure 1: *strip* gene analysis

A. The *strip* gene span (rearranged from Flybase). Transcription from the *strip* locus produces three different polypeptides. Grey boxes: non-coding exons; blue boxes: coding exons; lines: introns. On the top, yellow bars indicate the position of the amplicons used to produce RNAi transgenic lines. On the bottom, an upward triangle indicates the position of the P{EPg}HP37079 element insertion (Bloomington #22130). Non-precise excision of this P element gave rise to a *strip* null mutant ($\Delta 22130$): a red line indicates the region spans by the deletion. **B. Left** Anti-PDF staining in brains of adult *tim>strip-R1* and relative controls. PDF⁺ neurons harbor slightly abnormally in the medulla of RNAi-expressing flies. **Right** Anti-PDF staining in brains of larvae deleted for the *strip* gene ($\Delta 22130$) and relative controls. **C.** *strip* mRNA quantities in head extracts of *tim>strip-R1* and control flies collected every 3h at DD1. Results indicate the average value from two independent experiments and are normalized to the higher control value set to 100. Error bars indicate s.d.

Supplemental Figure 2: *Cka* gene analysis

A. The *Cka* gene span (rearranged from Flybase). Transcription from the *Cka* locus produces seven different polypeptides (Flybase). Grey boxes: non-coding exons; blue boxes: coding exons; lines: introns. On the top, yellow bars indicate the position of the amplicons used to produce RNAi transgenic lines. On the bottom, an upward triangle indicates the position of the P{PZ}Cka(05836) element insertion responsible for the *Cka*² protein null phenotype. **B.** Anti-PDF staining in brains of adult *tim>Cka-R1* and relative controls (**Left**), and in brains of homozygous *Cka*² adult mutants compared to heterozygous siblings (**Right**). **C.** *Cka* mRNA quantities in head extracts of *tim>Cka-R1* and control flies collected every 3h at DD1. Results indicate the average value from three independent experiments and are normalized to the higher control value set to 100. Error bars indicate s.e.m. **D.** Western blot showing the lack of anti-Cka staining in brain extracts of homozygous *Cka*² adults (**Right**). Wild-type flies (*w*;;) were tested for the Cka protein oscillation in brain extracts.

Supplemental Figure 3: *strip* and *Cka* effects on CLK

Western blot of head extracts of flies collected during the first day of constant conditions after 4 days of LD entrainment at 25°C. For each time point, extracts from control flies (C) are compared to extracts from *tim>strip*-RNAi (s), *tim>Cka*-RNAi (c) and *tim>strip*-RNAi, *Cka*-

RNAi (cs) flies. Black and grey rectangles indicate the subjective night and subjective day phases, respectively. Time (h) is indicated as CT. P-CLK: hyper-phosphorylated CLK.

Supplemental Figure 4: *strip* protein complexes

A. *Drosophila* S2 cells were transfected with a *pMt-strip-FgHA* plasmid (*dSp* - 400 ng) or an empty vector (-). Cell lysates were subjected to anti-HA immunoprecipitation (anti- β gal was used as a negative control). **B.** Anti-Flag immunoprecipitation (IP) from head extracts of *tim>Fg-strip* flies and *tim>+* negative controls at DD1. Supernatant and immunocomplexes were run into a gel. Transferred membranes were incubated with anti-WDB and anti-TWS antibodies.

Supplemental Figure 5: *wdb* gene analysis

A. The *wdb* gene span (rearranged from Flybase). Transcription from the *wdb* locus produces six different polypeptides (Flybase). Grey boxes: non-coding exons; blue boxes: coding exons; lines: introns. On the top, yellow bars indicate the position of the amplicon used to produce the TRiP RNAi transgenic line used in this study. **B.** Anti-PDF staining in brains of adult *tim>wdb-R1* and relative controls **C.** *wdb* mRNA quantities in head extracts of *tim>wdb-R1* and control flies collected every 3h at DD1. Results indicate the average value from two independent experiments and are normalized to the higher control value set to 100. Error bars indicate s.d. **D.** S2 cells were transfected with *pAct-Clk-V5* (400 ng) and 100 ng of *pAct-dbt-V5* or equal amounts of an empty vector. *pAct-GFP* transfection was used as an internal control for transfection efficacy. Cells were treated with dsRNA against *wdb* or *Cka* or *luc* (Luciferase: negative control) for 48h before lysis. Immunoblots are representative of two independent experiments.

Supplemental Figure 6: analysis of *mts*^{DN} phenotypes

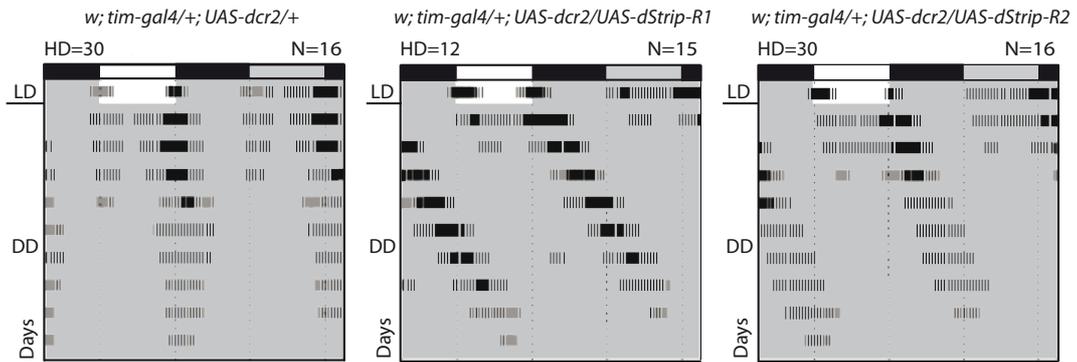
A. Representative actogram records showing the average locomotor activity of *tim>mts*^{DN} and control *tim>+* flies. One day of 12h:12h LD condition is followed by 9 days of constant darkness (DD) at 25°C. A shaded background indicates dark phase. On the top of the graph, black rectangles indicate night phases, while white and grey rectangles indicate day and subjective day phases, respectively. The hash density (HD: number of activity event per hash) varies according to the genotype (see Experimental procedures). **B.** Anti-PDF staining in brains of adult *tim>mts*^{DN} and control *tim>+* flies.

Table 1

Genotype	Number ^a	% R ^b	Avg period (h) ± s.e.m.	Power ^c
w; ; gal1118/+	16	100	24,1 ± 0,06	218,8
w; ; gal1118/UAS-strip-R1	16	100	26,0 ± 0,10	128,8
w; ; UAS-strip-R1/+	16	93,8	23,9 ± 0,06	206,0
w; ; UAS-strip-R2/+	16	100	23,5 ± 0,06	209,4
w; UAS-Cka-R1/+;	32	93,8	23,4 ± 0,04	195,0
w; ; UAS-Cka-R5	23	100	23,3 ± 0,06	203,2
w; ; UAS-wdb-R1/+	29	100	23,6 ± 0,05	192,7
w; ; #22130	11	100	23,8 ± 0,10	134,2
w; ; #22130/Df	16	100	24,4 ± 0,10	224,2
w; Cka ² , HS-Cka/CyO;	18	83,3	24,6 ± 0,23	100,9
w; Cka ² , HS-Cka;	14	35,7	26,9 ± 2,30	49,6
w; UAS-dcr2/+; gal1118/+	16	100	24,0 ± 0,05	215,6
w; UAS-dcr2/+; gal1118/UAS-strip-R1	16	100	26,4 ± 0,10	147,5
w; UAS-dicer2/+; gal1118/UAS-strip-R2	16	100	25,3 ± 0,06	279,5
w; UAS-dcr2/UAS-Cka-R1; gal1118/+	16	100	25,5 ± 0,07	264,2
w; UAS-dicer2/+; gal1118/UAS-Cka-R5	16	100	24,9 ± 0,04	284,2
w; UAS-dcr2/+; gal1118/UAS-wdb-R1	16	100	25,0 ± 0,06	302,4
w; timgal4/+; UAS-dcr2/+	16	100	23,9 ± 0,08	177,9
w; timgal4/+; UAS-dcr2/UAS-strip-R1	16	87,5	26,6 ± 0,12	125,5
w; timgal4/+; UAS-dicer2/UAS-strip-R2	16	100	25,7 ± 0,06	197,0
w; timgal4/UAS-Cka-R1; UAS-dcr2/+	16	100	25,9 ± 0,06	198,7
w; timgal4/+; UAS-dicer2/UAS-Cka-R5	16	100	25,5 ± 0,10	135,9
w; timgal4/+; UAS-dcr2/UAS-wdb-R1	16	100	25,1 ± 0,06	218,1
w; timgal4/UAS-Cka-R1; UAS-dcr2/UAS-strip-R1	29	69	26,7 ± 0,10	68,0
w; timgal4/UAS-dcr2; UAS-strip-R1/UAS-wdb-R1	13	92,3	28,2 ± 0,14	72,1
w; timgal4/UAS-Cka-R1; UAS-dcr2/UAS-wdb-R1	16	100	26,5 ± 0,06	188,2
w; timgal4/UAS-dcr2; UAS-dbt ^{K/R} , dbt ^{ar} /+	16	6,3	36,0 ± 0,00	75,0
w; timgal4/UAS-Cka-R1; UAS-dbt ^{K/R} , dbt ^{ar} /UAS-dcr2	16	25	40,3 ± 0,43	41,0
w; timgal4/UAS-dcr2; UAS-dbt ^{K/R} , dbt ^{ar} /UAS-strip-R1	16	25	28,0 ± 3,01	27,0
w; timgal4/+; UAS-mts ^{DN} /+	16	31,3	27,3 ± 3,18	20,3

Figure 1

A



B

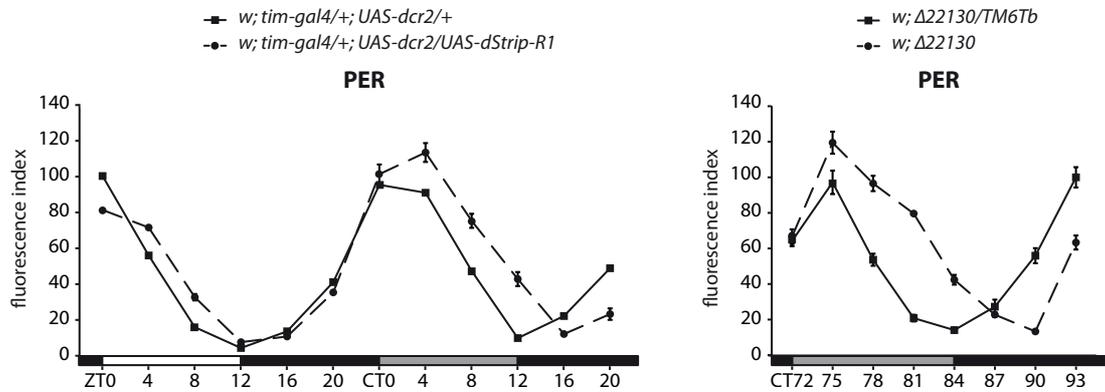
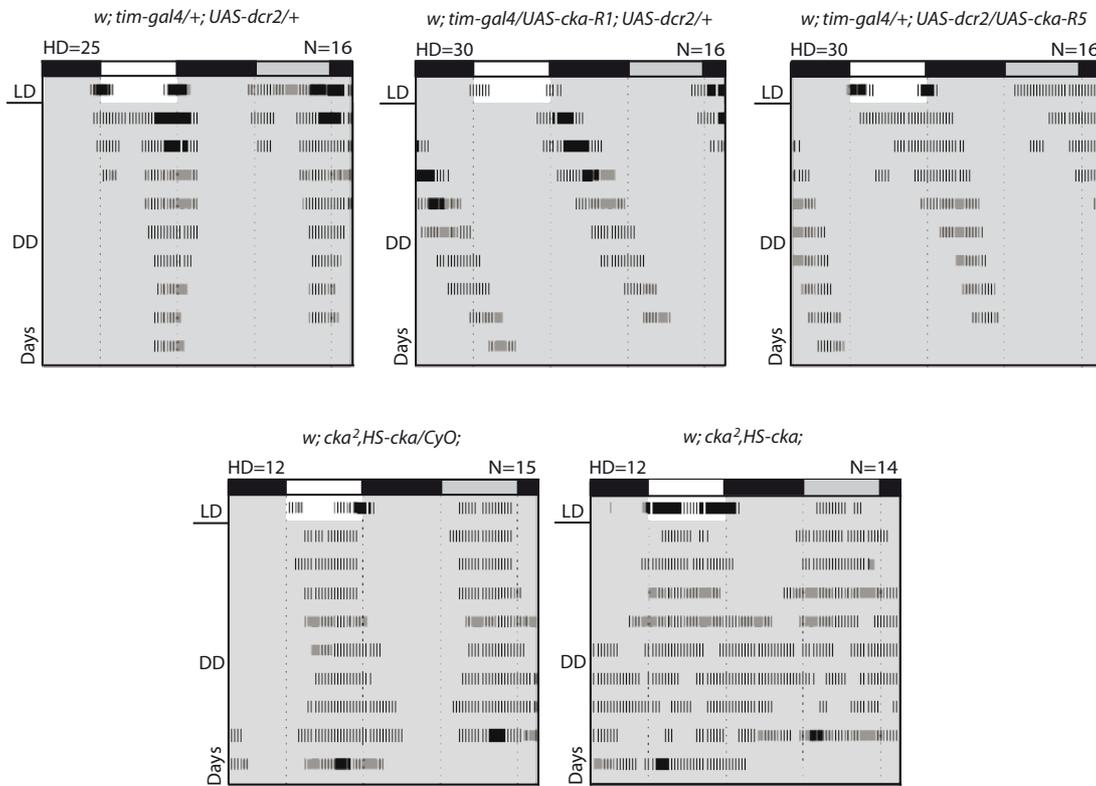


Figure 2

A



B

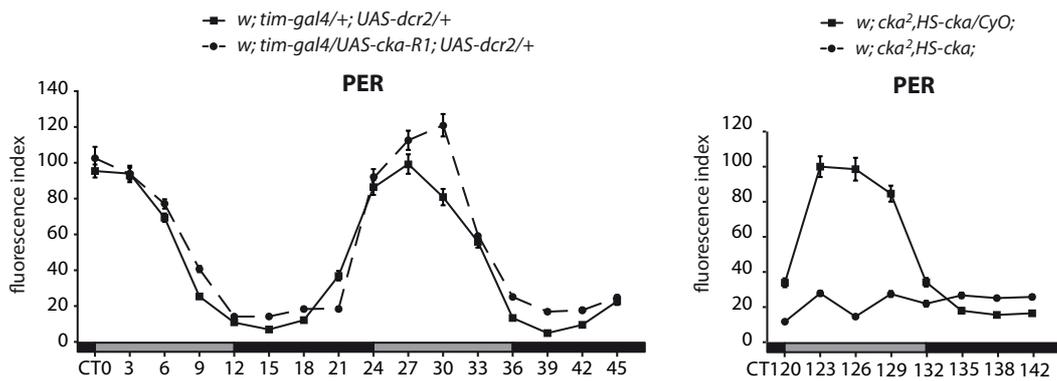


Figure 3

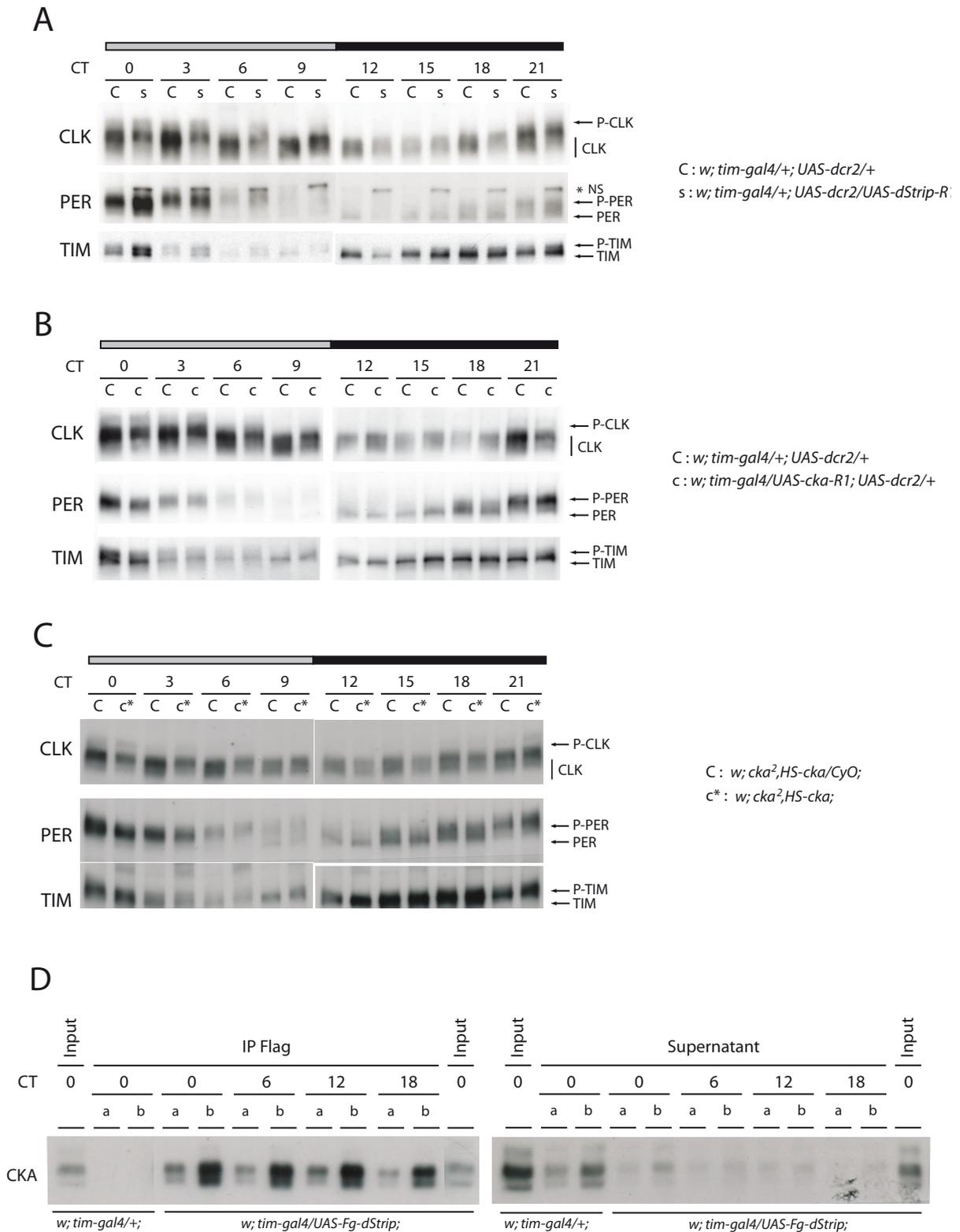
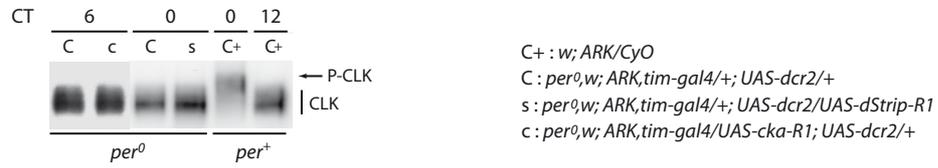
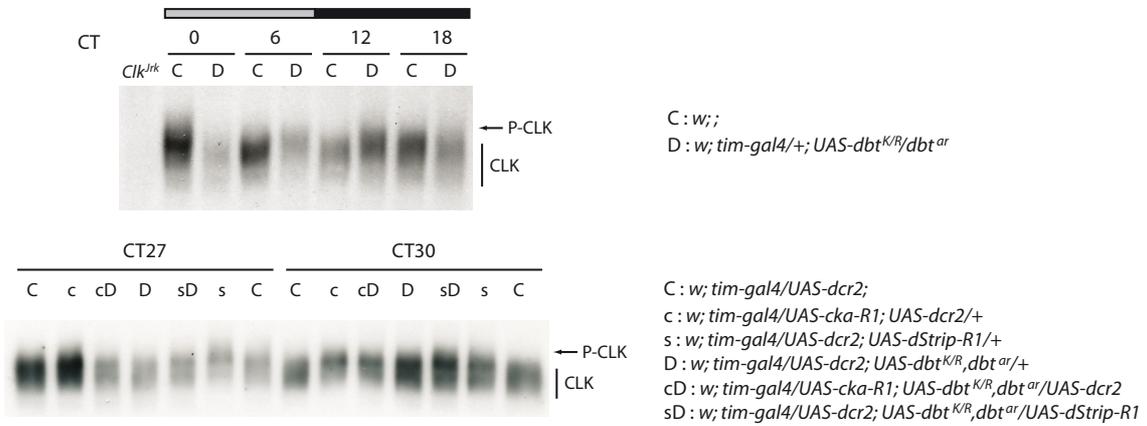


Figure 4

A



B



C

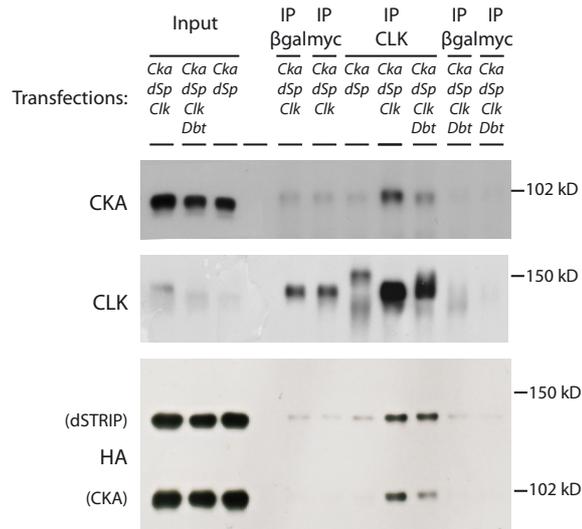


Figure 5

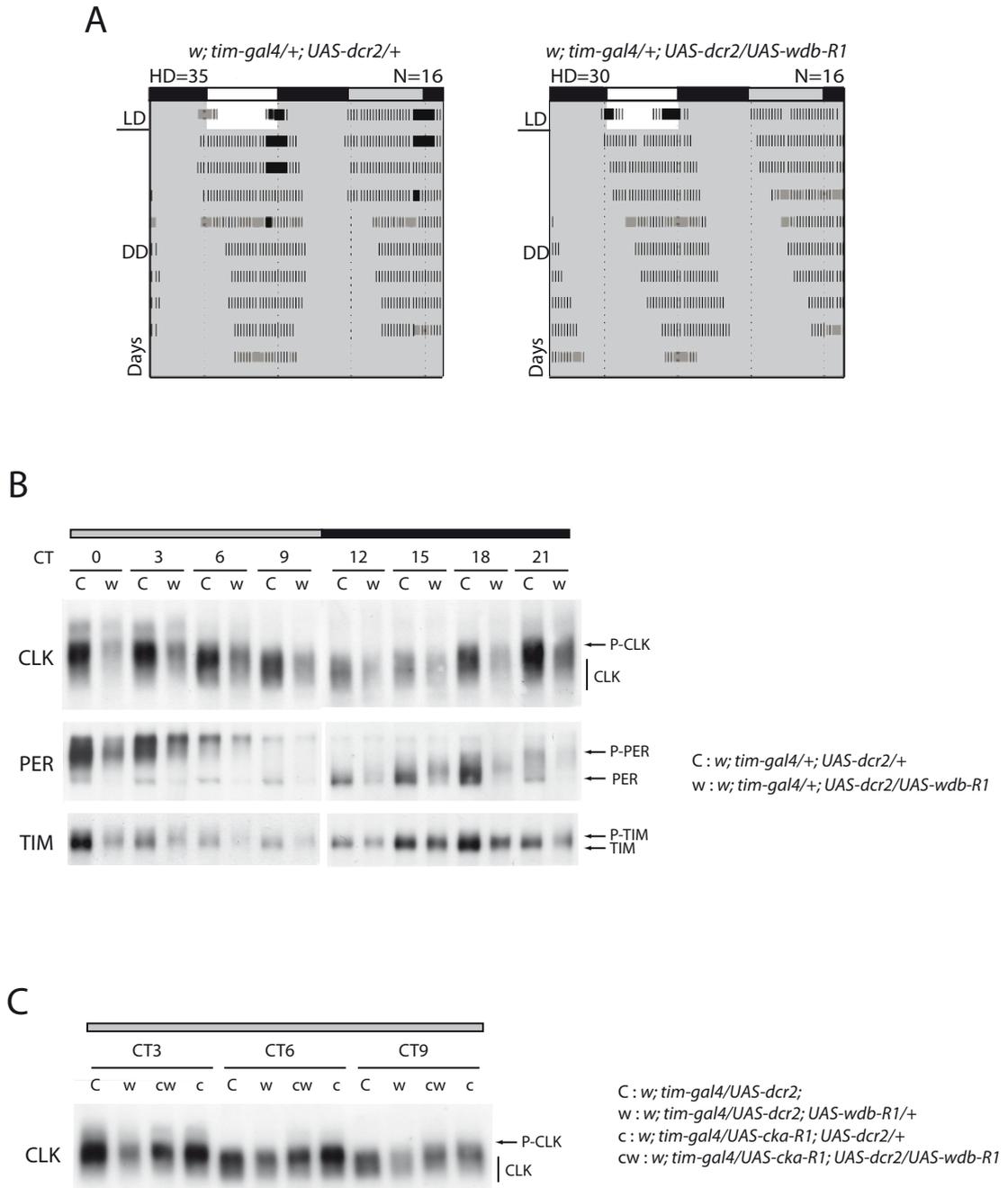


Figure 6

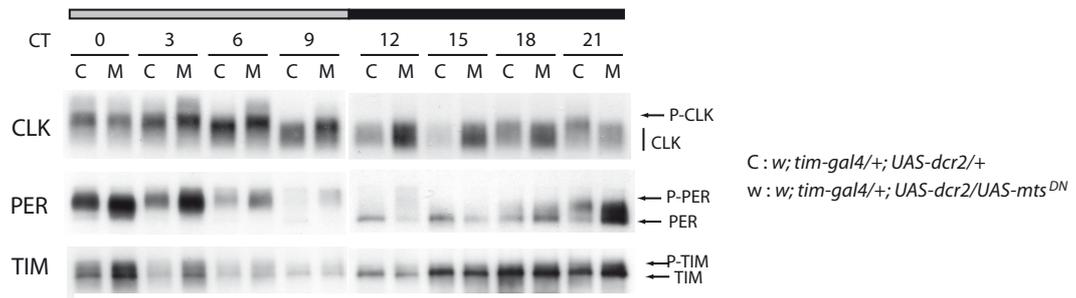
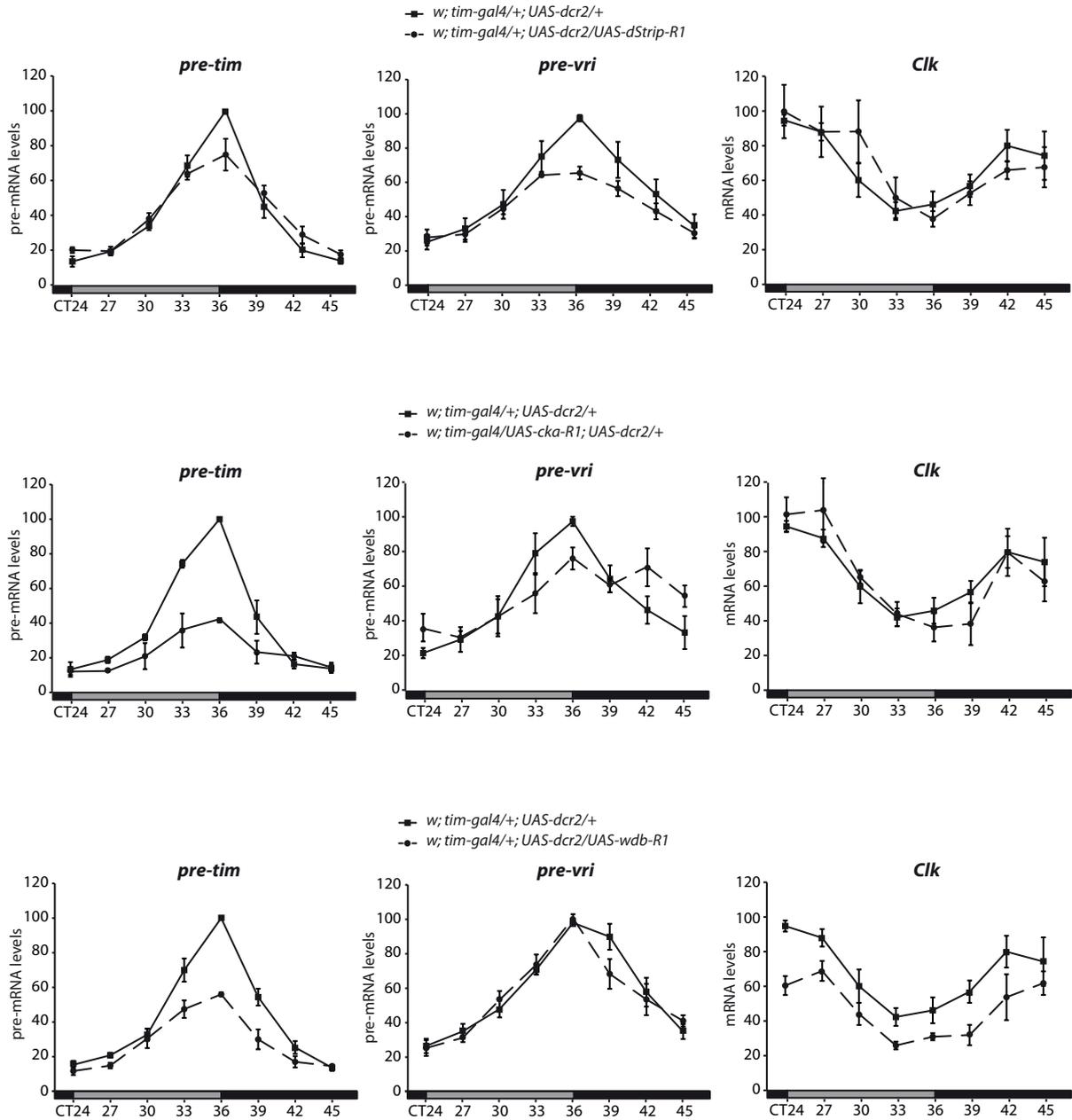


Figure 7

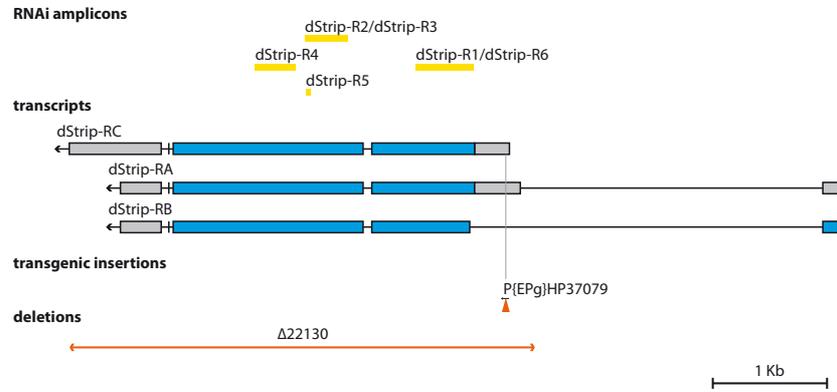


Supplemental Table 1

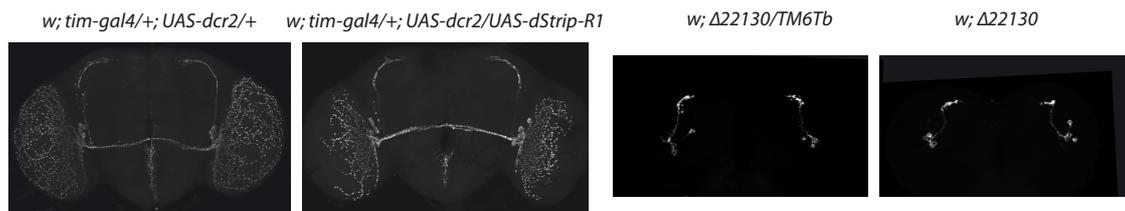
Genotype	Number ^a	% R ^b	Avg period (h) ± SEM	Power ^c
w; UAS-dicer2/+; gal1118/+	16	100	24,0 ± 0,05	215,6
w; UAS-dicer2/+; gal1118/strip-R3	16	100	25,4 ± 0,09	257,9
w; UAS-dicer2/strip-R4; gal1118/+	16	100	25,2 ± 0,11	251,1
w; UAS-dicer2/+; gal1118/strip-R5	16	100	25,4 ± 0,06	271,4
w; UAS-dicer2/+; gal1118/strip-R6	16	100	25,4 ± 0,12	234,8
w; pdfgal4/+	16	100	24,2 ± 0,14	179,9
w; pdfgal4/+; strip-R1/+	16	100	26,1 ± 0,14	155,5
w; timgal4/+;	16	100	23,9 ± 0,05	241,8
w; timgal4/+; strip-R1/+	16	100	26,0 ± 0,09	144,3
w; timgal4/+; UAS-dicer2/+	16	100	23,9 ± 0,08	177,9
w; timgal4/+; UAS-dicer2/strip-R3	16	93,3	25,7 ± 0,08	177,2
w; timgal4/strip-R-4; UAS-dicer2/+	16	100	25,4 ± 0,06	181,5
w; timgal4/+; UAS-dicer2/strip-R5	16	100	25,3 ± 0,06	209,1
w; timgal4/+; UAS-dicer2/strip-R6	16	100	25,5 ± 0,05	180,0
w; clkgal4/Y; ;	16	100	23,7 ± 0,08	213,6
w,clkgal4/Y; ; strip-R1/+	16	100	25,3 ± 0,08	169,9
w,clkgal4/Y; +/CyO; UAS-dicer2/+	11	100	23,3 ± 0,08	151,9
w,clkgal4/Y; +/CyO; UAS-dicer2/strip-R1	16	93,8	26,1 ± 0,08	146,2
w; ; gal1118/+	16	100	24,0 ± 0,06	195,9
w; cka-R1/+; gal1118/+	16	100	25,6 ± 0,08	248,9
w; cka-R2/+; gal1118/+	16	100	25,7 ± 0,08	263,7
w; cka-R3/+; gal1118/+	15	100	25,4 ± 0,06	278,1
w; ; gal1118/cka-R4	16	100	24,2 ± 0,16	232,8
w; ; gal1118/cka-R5	16	100	25,1 ± 0,10	262,6
w; UAS-dicer2/+; gal1118/+	15	100	24,0 ± 0,03	224,2
w; UAS-dicer2/cka-R2; gal1118/+	16	100	25,7 ± 0,06	236,0
w; UAS-dicer2/cka-R3; gal1118/+	16	100	25,2 ± 0,06	271,1
w; UAS-dicer2/+; gal1118/cka-R4	16	100	24,7 ± 0,22	254,8
w; timgal4/+;	16	100	23,8 ± 0,06	248,4
w; timgal4/cka-R1;	16	100	25,7 ± 0,06	269,6
w; timgal4/cka-R2;	16	100	25,7 ± 0,06	232,3
w; timgal4/cka-R3;	15	100	25,4 ± 0,05	280,0
w; timgal4/+; cka-R4/+	16	100	24,0 ± 0,15	240,8
w; timgal4/+; cka-R5/+	16	93,8	25,1 ± 0,05	251,5
w; timgal4/+; UAS-dicer2/+	16	100	23,7 ± 0,08	201,3
w; timgal4/cka-R2; UAS-dicer2/+	16	100	25,9 ± 0,07	212,8
w; timgal4/cka-R3; UAS-dicer2/+	16	93,8	25,6 ± 0,10	204,2
w; timgal4/+; UAS-dicer2/cka-R4	16	93,8	25,2 ± 0,11	123,6
w; ; gal1118/+	15	100	23,9 ± 0,05	241,3
w; ; gal1118/wdb-R1	16	100	24,4 ± 0,07	276,2
w; timgal4/+;	16	100	24,1 ± 0,08	231,2
w; timgal4/+; wdb-R1/+	16	100	24,3 ± 0,06	229,4

Supplemental Figure 1

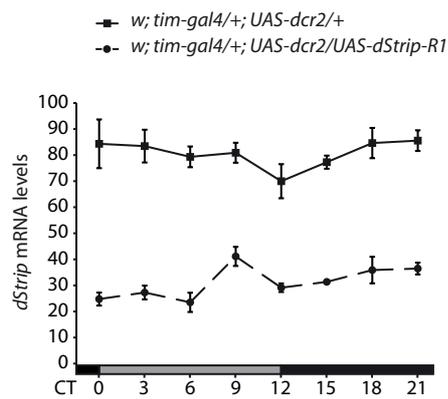
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B

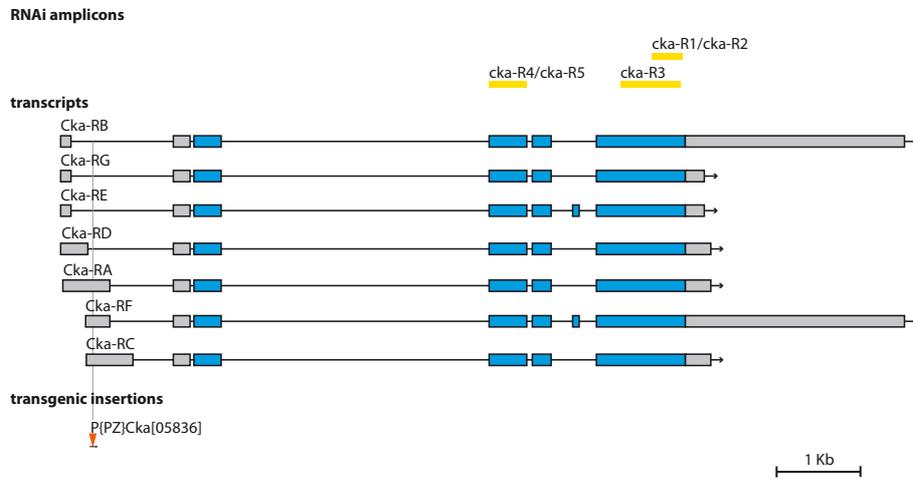


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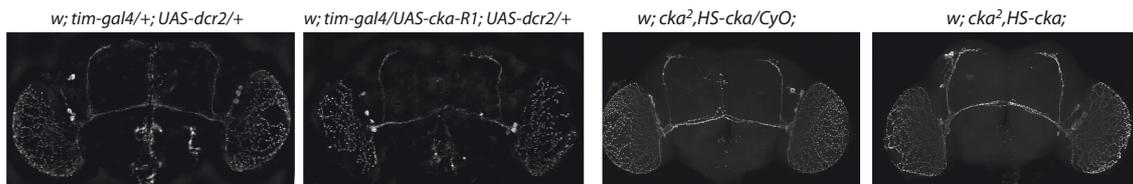


Supplemental Figure 2

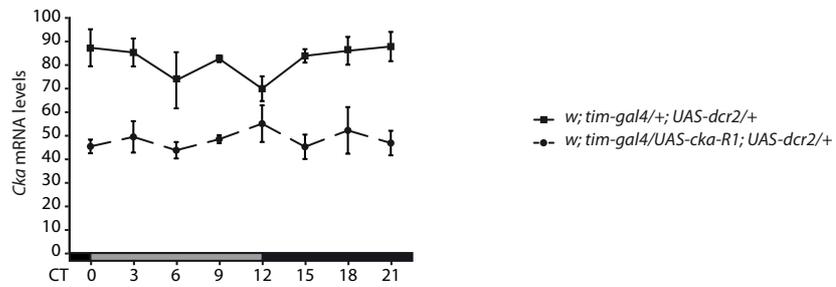
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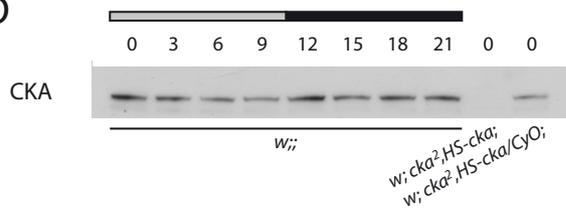
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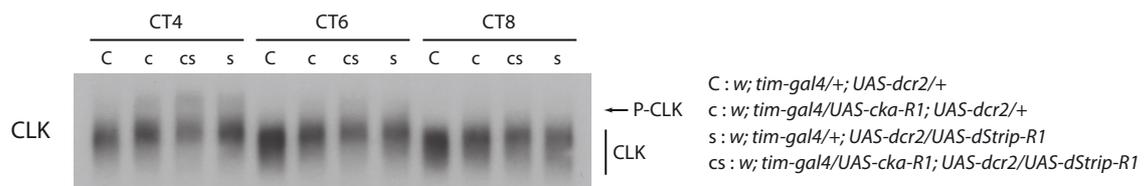
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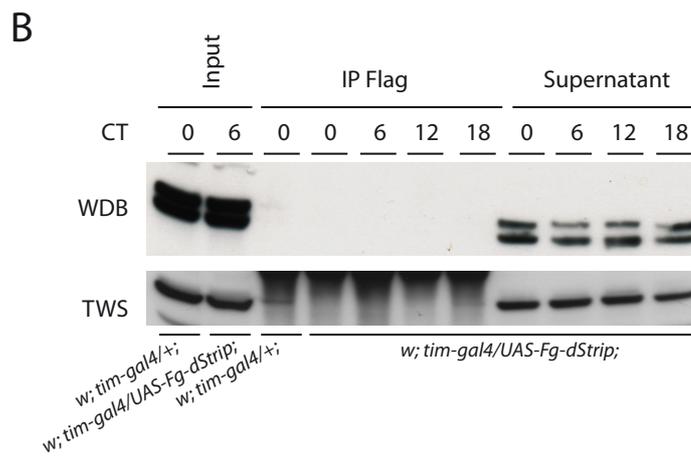
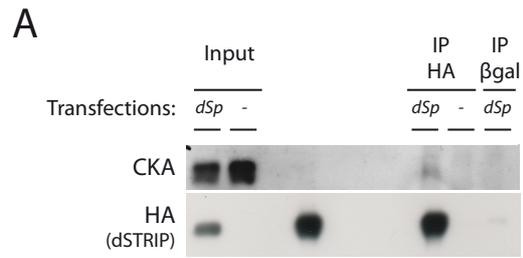
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Supplemental Figure 3

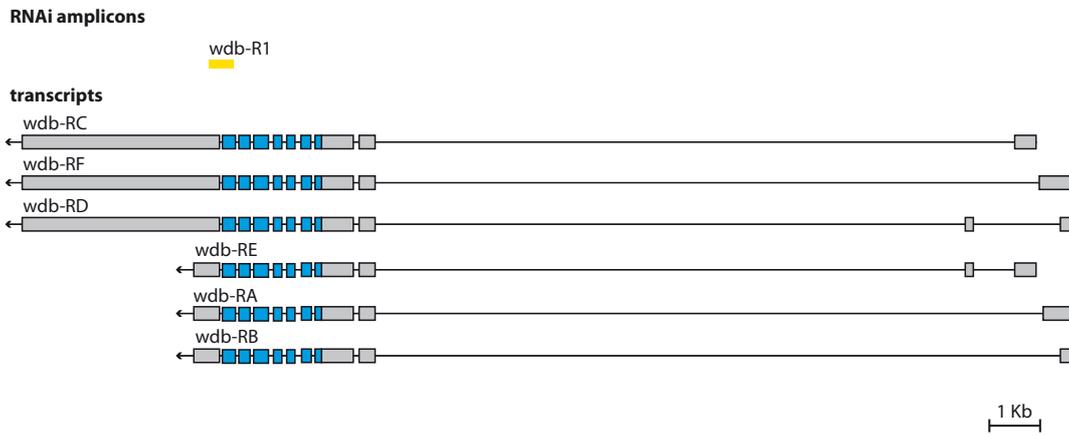


Supplemental Figure 4

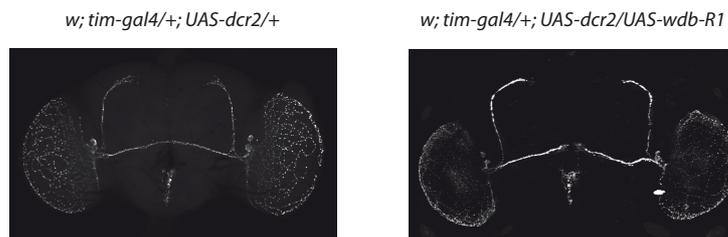


Supplemental Figure 5

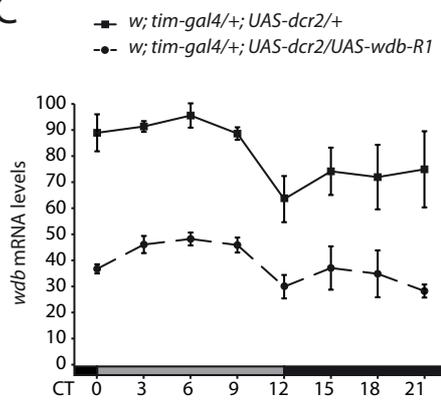
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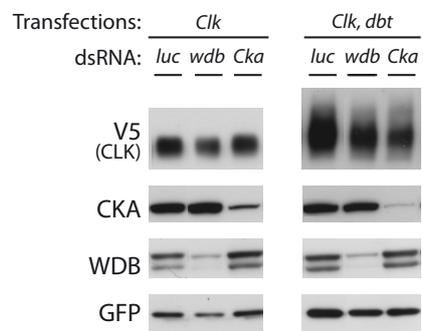
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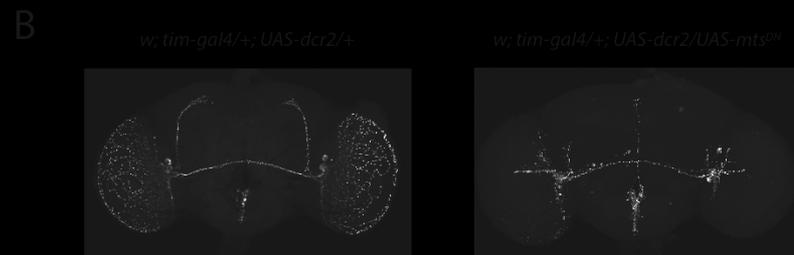
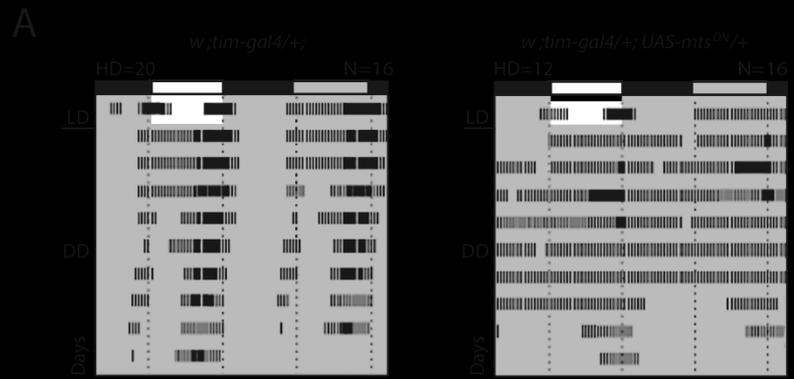
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Supplemental Figure 6



2. Unpublished data

a. Strip alters CLK localization

In cultured S2 cells the CLK protein can be detected both in the nucleus and in the cytoplasmic compartments (Hung et al., 2009), in agreement with the presence of specific NLS and NES motifs in the CLK protein sequence. However, the CLK staining in neurons is largely restricted to cell nuclei at all times of the daily cycle (Houl et al., 2006) and we cannot detect cytoplasmic CLK by *in situ* studies. Indeed, fractionation data from fly head extracts indicated that the CLK protein may be found in the cytoplasm compartment also *in vivo* (Hung et al., 2009). By the use of this technique, we checked the localization of CLK in wild-type flies and in *strip*-RNAi flies.

Drosophila wild-type flies were collected at different time points during standard 12h:12h LD conditions and heads were subjected to fractionation analysis to subdivide the nuclear and cytoplasmic components, and the resulting extracts were separately blotted (Fig. 1A). Westerns revealed that the CLK protein shuttled between a mainly cytoplasmic and mainly nuclear localization, respectively at the night-to-day and the day-to-night transitions. CLK started to accumulate in the cytoplasm early in the morning and then moved to the nucleus, where it reached its maximum quantity towards the middle of the night. These data fitted with times of CLK-mediated transcriptional activation and repression.

We next investigated if and how localization of the CLK protein was altered in *strip* downregulated flies. Flies expressing the *strip*-RNAi in *tim*⁺ cells were collected at two opposite time points (ZT3 and ZT15) and head extracts were subdivided into separate nuclear and cytoplasmic pools (Fig. 1B). CLK localization was altered at both time points: higher CLK levels in the cytoplasm and lower CLK staining in the nucleus were seen in RNAi extracts compared to wild-type controls (Fig. 1B). Effects were even stronger when we normalized the signal using compartment-specific markers (Tubulin, for cytoplasmic fraction, and Histone, for nuclear fraction). In agreement with *strip*-RNAi effects previously shown, the CLK staining appeared upward shifted (higher molecular weight) in RNAi expressing extracts compared to controls. Strip thus regulates, directly or indirectly, the ratio between nuclear and cytoplasmic CLK and/or CLK nuclear-cytoplasm transport, and promotes CLK de-phosphorylation.

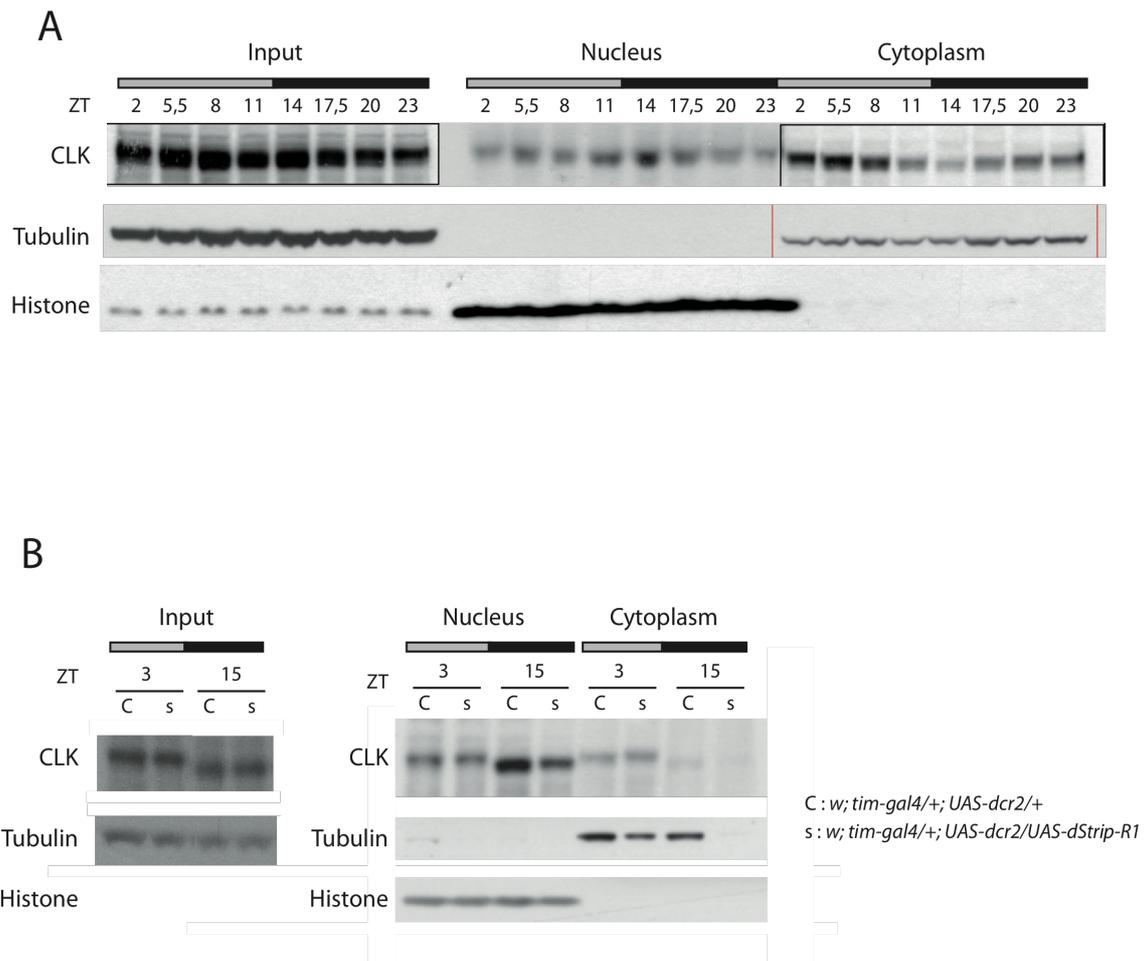


Fig. 1 Strip controls nuclear localization of the CLK protein

A. Fractionation data showing staining of the CLK protein in the nucleus and cytoplasmic fractions of wild-type fly head extracts collected every 3h during standard LD conditions at constant 25°C temperature. CLK is more abundant in the nucleus at the beginning of the night, while CLK quantities are higher in the cytoplasm during the morning. Anti-Tubulin staining is used as a loading control for cytoplasmic fraction, while anti-Histone H3 labels nuclear fraction at all time points. **B.** *strip*-RNAi expression favors higher CLK staining in the cytoplasm and lower CLK quantities in the nucleus. CT3 and CT15 are analyzed. Tubulin and Histone staining are used as loading control.

b. Strip-containing complexes possess some Cka-independent functions

The downregulation either of *Cka* or *strip* affected the molecular profile of the CLOCK protein. In addition to CLK, expression of the *strip*-RNAi also altered the stability of the PERIOD and TIMELESS proteins. In fact, TIM and especially PER staining persisted longer during the morning in *strip*-RNAi flies, unlike the scenario in wild-type control, in which phosphorylated TIM and PER undergo rapid degradation early during the daytime.

Importantly, these effects were not seen for *Cka* downregulation and were thus specific to *strip* knockdown.

The PER protein was stabilized upon *strip*-RNAi induction also in a *tim*⁰ background (Fig. 2B). In addition, the hypo-phosphorylated PER forms that accumulated in *dbt*^{K/R} flies were further stabilized when *strip* was downregulated (Fig. 2A). Altogether, these data suggest that Strip or a Strip-containing complex participates in the regulation of PER protein quantities.

In both a *tim*⁰ and a *dbt*^{K/R} genetic backgrounds, the PER protein fails to attain its higher phosphorylation state and localizes mainly in the cytosol (Price, 1995; Muskus et al., 2007). Therefore, Strip favors the destabilization of non-DBT-phosphorylated PER forms that accumulate in the cytoplasm. TIM is thought to protect PER from degradation. Our results demonstrated that Strip is involved in the destabilization of PER in the absence of TIM (*tim*⁰). In addition, *strip* destabilized PER also in the presence of TIM, but at times (in the morning) when TIM was thought to be no more bound to PER. In conclusion, Strip destabilizes PER not bound to TIM.

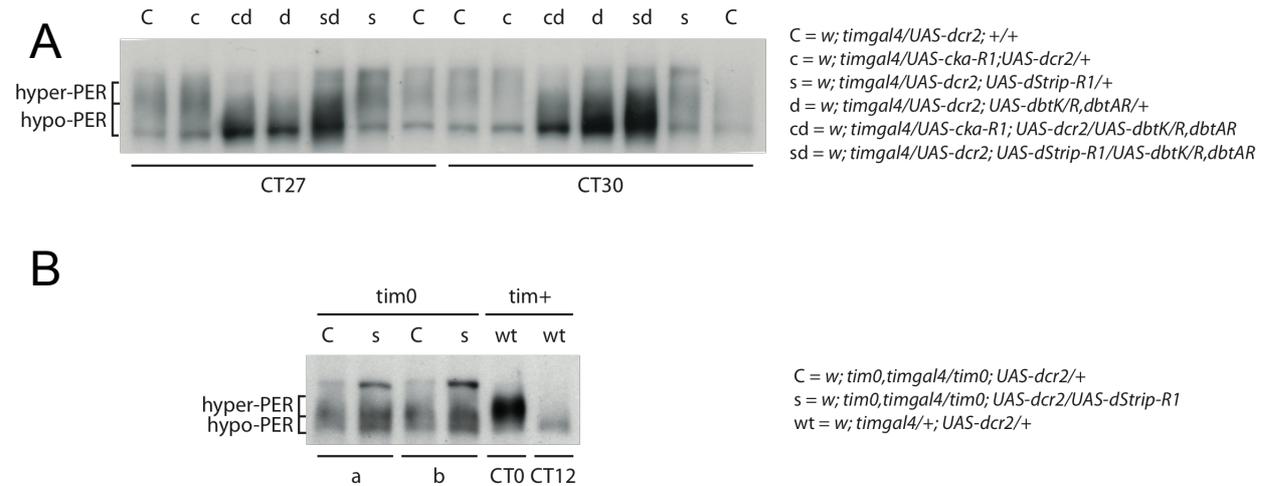


Fig. 2 Strip controls PER degradation independently of Cka

A. Western blot of head extracts of flies depleted for *strip* (s), *Cka* (c), *Dbt* functions (d) and a combination of these genotypes. *strip*-RNAi expression stabilizes PER, either in a wild-type *Dbt* or in a hypomorph *Dbt* genetic background (*dbt*^{K/R}, *dbt*^{ar}). **B.** PER stabilization by *strip*-RNAi expression also occurs in a *timeless* null background (*tim*⁰). *a* and *b* indicate extracts from two different experiments (technical replicate).

c. CLK phosphorylation by the NEMO kinase is required for PP2A/Cka activity on CLK

Phosphorylated CLK is the expected substrate for the PP2A phosphatase activity. The CLK protein undergoes circadian cycles in phosphorylation: CLK phosphorylation is maximal in the morning and minimal in the evening. Yet, very little is known about the kinases that affect CLK phosphorylation. In addition to DBT, casein kinase 2 – alpha subunit (CK2 α) and Nemo kinase (Nmo) were shown to affect CLK phosphorylation and stability (Yu et al., 2011; Szabo et al., 2013).

We used the overexpression of a dominant negative form (CK2 α^{Tik}) to largely titrate CK2 α activity in clock cells. *tim>CK2 α^{Tik}* flies were rhythmic but adopted a very long period (>30h). The period was even longer when *Cka*-RNAi was added to the CK2 α^{Tik} background, suggesting that the two proteins independently affected the circadian activity of flies (Table 1). As shown in Fig. 3A, overexpression of CK2 α^{Tik} together with *Cka*-RNAi favored additional post-translational modifications to occur on the hyper-phosphorylated CLK that accumulated in the morning. A possible scenario is that the CLK non-phosphorylated by CK2 α is recognized by other kinase(s), whose phosphorylation activity is counterbalanced by PP2A/Cka. In addition, *Cka*-RNAi did not modify CK2 α^{Tik} -induced molecular effects on the PER and TIM proteins.

Work from P. Hardin (Yu et al., 2011) showed that downregulation of the Nemo kinase (*nmo*) by RNAi induces short-period rhythms of about 22h, which were accompanied by higher CLK abundance and phosphorylation. *nmo^P* is a severely hypomorphic *nmo* mutant that shows behavioral phenotypes (*nmo^P/nmo*-deficiency: *nmo^P/Df*) very similar to those induced by the expression of *nmo*-RNAi (Table 2). We tested the effects of *Cka*-RNAi in a *nmo^P/Df* background. For *nmo* downregulation, we preferred to use mutants instead of RNAi in order to avoid possible titration effects caused by the presence of multiple copies of UAS-constructs and only one copy of the driver. First, we wanted to reproduce the *nmo*-RNAi phenotype with *nmo^P* mutants (Fig. 3C). In contrast to what was observed with RNAi, in a *nmo^P/Df* background the CLK protein did oscillate as in wild-type controls, yet CLK quantities were reduced at all time points. Combining the short-periodicity of *nmo* mutants with the long-period phenotypes of *Cka*-RNAi flies (*tim>Cka-RNAi;nmo^P/Df*) allowed the rescue of *quasi* normal rhythm of 24h (Table 1). Western blots revealed that when Nemo functions were compromised in addition to *Cka*, the hyper-phosphorylated CLK forms that normally appeared after *Cka* downregulation were missing (Fig. 3B). Moreover, in extracts

from *tim>Cka-RNAi;nmo^P/Df* flies, the molecular oscillations of the CLK protein were similar to those shown by wild-type controls, but CLK quantities were severely reduced, even compared to *nmo^P/Df* single mutants.

Altogether, data are consistent with Nemo working upstream of Cka to produce a suitable substrate for PP2A/Cka-mediated CLK dephosphorylation and stabilization.

Genotype	Number ^a	% Rhythmicity ^b	Avg period (h) ± SEM	Power ^c
<i>w; timgal4/+;</i>	31	100	23,9 ± 0,05	219,0
<i>w; timgal4/ UAS-CKA-R1;</i>	32	96,9	25,7 ± 0,04	198,1
<i>w; timgal4/+; nmo^{P1}/nmo^{Df}</i>	24	91,7	21,9 ± 0,09	120,8
<i>w; timgal4/ UAS-CKA-R1; nmo^{P1}/nmo^{Df} *</i>	12	100	24,6 ± 0,10	152,5
<i>w; timgal4/+; UAS-dcr2/+</i>	32	96,9	23,8 ± 0,05	179,0
<i>w; timgal4/UAS-CKA-R1; UAS-dcr2/+</i>	32	100	26,0 ± 0,05	244,7
<i>w; timgal4/+; UAS-dcr2/UAS-Tik</i>	32	100	32,6 ± 0,06	198,7
<i>w; timgal4/UAS-CKA-R1; UAS-dcr2/UAS-Tik</i>	31	100	35,1 ± 0,08	180,0
<i>w; UAS-dcr2/+; gal1118/+</i>	16	100	24,1 ± 0,06	287,1
<i>w; UAS-dcr2/+; gal1118/UAS-MSN-R1</i>	16	38	22,7 ± 0,17	99,37
<i>w; UAS-dcr2/UAS-GCKIII-R1; gal1118/+</i>	16	100	24,8 ± 0,06	240,6

Table 1 Locomotor activity of flies downregulated for known and putative CLK kinases

Flies were kept at 25°C and exposed to at least 2 days of 12:12 LD followed by 11 days of DD conditions. Rhythmicity is calculated using the last 10 days of experiment. Results are referred to male flies, except for *nmo*, *Cka* downregulated flies (*) that are females. CKA-R1: VDRC #35234; Tik: *CK2α^{Tik}*, dominant negative transgenic allele for *CK2α*. *nmo^{P1}*: severe hypomorph allele for *nmo*; *nmo^{Df}*: deficiency covering the *nmo* locus. MSN-R1: NIG-FLY #16973-R1; GCKIII-R1: VDRC #22024.

^a Total number of flies that survived until the end of the testing period

^b Percentage of flies with activity rhythms having a power value of ≥ 30

^c Measure of the strength or amplitude of the rhythm.

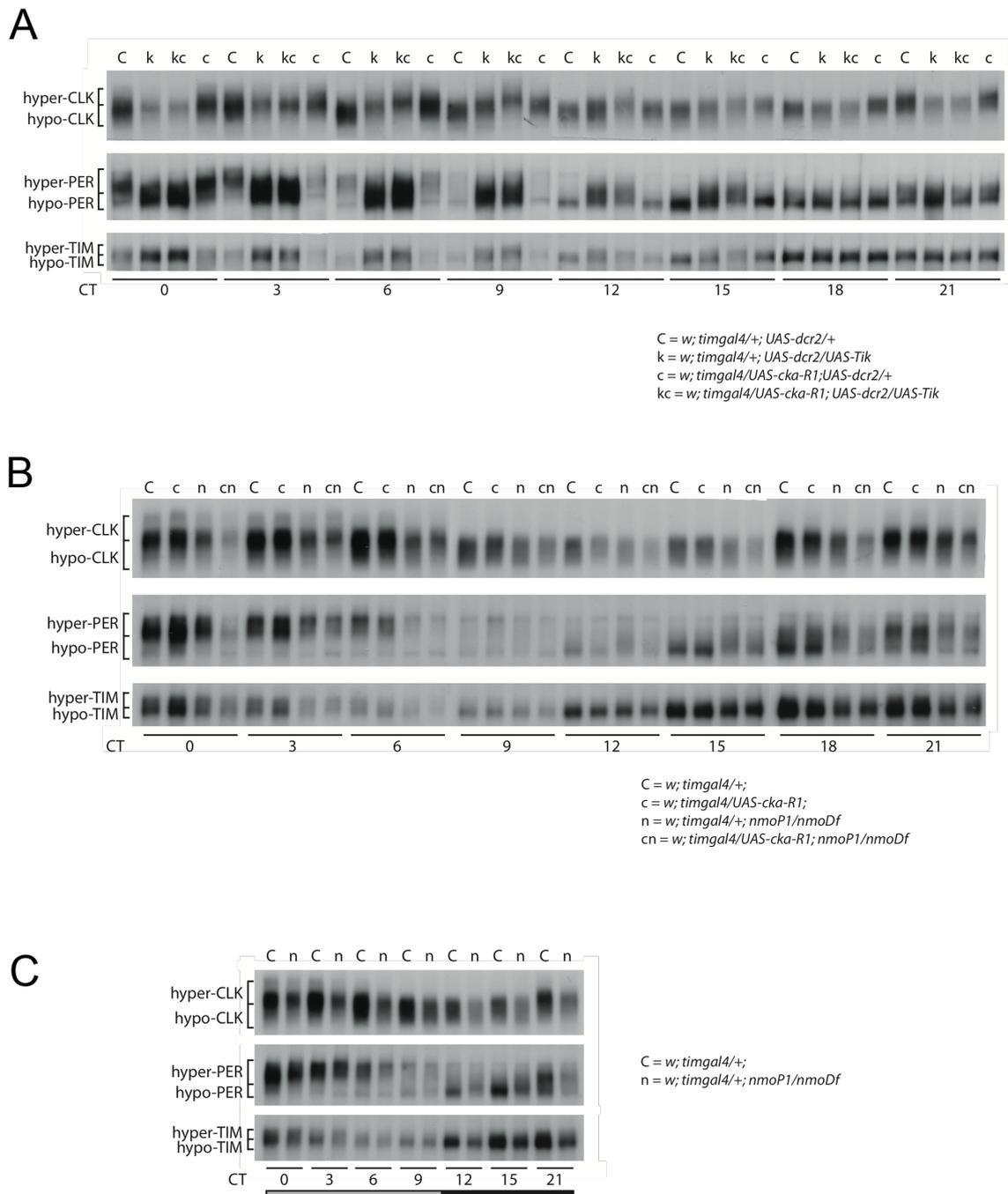


Fig. 3 CK2 and Cka play independent roles on CLK phosphorylation while Nemo-phosphorylated CLK is required for Cka-mediated dephosphorylation.

A. Western blot of head extracts of flies overexpressing a dominant negative form of CK2a (*Tik* mutants, k), Cka-RNAi (c) or both (kc) in *tim*-positive cells, compared to controls (C). Downregulation of both CK2 kinase functions and PP2A/Cka phosphatase activities triggers additional phosphorylation on the CLK protein in the middle of the day. **B.** Western blot of head extracts of flies expressing Cka-RNAi (c) in *tim*-positive cells, flies mutant for the Nemo kinase (n) or both (cn), compared to controls (C). The hyper-phosphorylated CLK phenotype of Cka-RNAi expressing flies is (at least partially) abrogated by mutations at the Nemo kinase. **C.** Hypomorph nemo mutants (n) show hyper-phosphorylated and less abundant PER and CLK proteins compared to controls (C).

d. Within the STRIPAK complex, PP2A/Cka associates with members of the Ste20 kinase family

In addition to the PP2A subunits, the STRIPAK complex contains other interacting proteins, including kinases (Fig. 4). Members of the Ste-20 kinase family, like Hippo (Hpo), Misshapen (Msn) or Germinal Center Kinase III (GckIII), co-immunoprecipitated with Cka in cultured cell assays (Ribeiro et al., 2010). We wondered whether downregulation of either of these kinases would also affect the pace of the fruit fly clock.

We tested different UAS-RNAi transgenes targeting the *Msn* or *GckIII* mRNAs and used the *gal1118*-Gal4 driver to direct kinase downregulation only to the s-LN_v neurons (Table 2). *gckIII* downregulation through expression of the VDRC 22024-RNAi line induced the flies to behave with a period of 25h in constant conditions (+1h compared to controls). Conversely, expression of the 16973-R1 RNAi (NIG-Fly collection), which targets *Msn*, led to arrhythmic behaviors. While preliminary, these data suggest that the Misshapen and GckIII kinases affect the mechanisms that control the pace of the *Drosophila* clock. Possibly, Msn and/or GckIII cooperate with PP2A/Cka within the STRIPAK complex to control phosphorylation/dephosphorylation of the CLK protein.

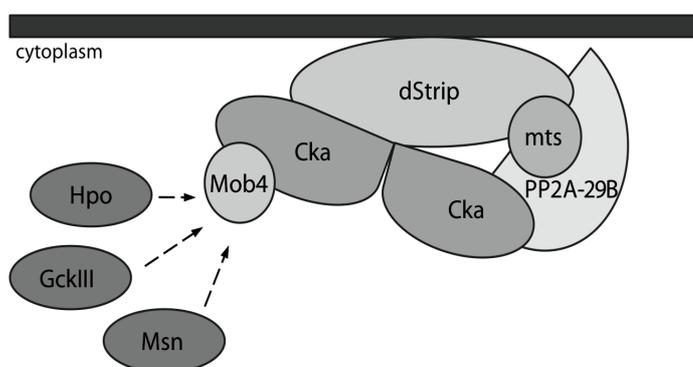


Fig. 4 Organization of the STRIPAK super-complex

The STRIPAK complex contains the PP2A catalytic (microtubule star = mts), scaffold (PP2A-29B) and the connector of kinase to AP-1 (Cka) regulatory subunits. Strip anchors the PP2A holoenzyme to the cytoplasm. Members of the Ste-20 family of protein kinases (Hippo = Hpo, Germinal center kinase III = GckIII, Misshapen = Msn) associate to the complex through the Mob4 protein.

Supplemental methods

Nuclear/cytoplasmic fractionation

Fly heads were put in a Dounce homogenizer, containing homogenization buffer (10mM HEPES pH7.5, 10mM KCl, 0.8M Sucrose and 1mM EDTA, with freshly added 0.5mM phenylmethylsulfonylfluoride (PMSF) and 0.5mM dithiothreitol (DTT)). After homogenization, the homogenate was filtered through a column polymer bed support (Bio-Rad unfilled Bio-spin Column) to remove the cuticle. The filtrate was then centrifuged, and the pelleted cell extract was then subjected to nuc/ cyt fractionation. To prepare the cytoplasmic fraction, the cell pellets were resuspended in cytoplasmic buffer (10 mM Tris HCl pH 8.0, 10 mM KCl). Cells were allowed to swell for 15 min, and then NP-40 was added to 0.4%, followed by centrifugation. The supernatant contained the soluble cytoplasmic fraction. The pellets were washed once more with the cytoplasmic buffer before proceeding to nuclear fractionation. For the preparation of the nuclear fraction, the remaining cell pellet was resuspended in high-salt buffer (50 mM Tris pH 8.0, 5mM EDTA, 400mM NaCl, 1% NP-40, 1% deoxycholate, and 0.025% sodium dodecyl sulfate (SDS), 1mM DTT). The nuclear pellet was hard vortex for 30 min at 40C. The purity of the cytoplasmic fraction was verified by probing with anti- α -tubulin antibody, while that of the nuclear fraction was verified with anti-histone 3 antibody. Nuclear and cytoplasmic extracts were subjected to Western blot analysis using the indicated antibodies.

3. Discussion

a. PP2A/CKA complexes control dephosphorylation of the CLK protein while PP2A/WDB affects global CLK quantity.

In the PP2A holoenzyme, regulatory subunits play a pivotal role in dictating the substrate specificity and the subcellular localization of the complex. Recently, CKA has been isolated as a novel PP2A B''-type regulatory subunit (Ribeiro et al., 2010).

CKA downregulation resulted in CLK hyper-phosphorylation both in adult flies (particularly during the day-time) and in insect S2 cells. Hyper-phosphorylated CLK appeared in fly head extracts also when we downregulated all PP2A activities using clock cell-specific overexpression of a dominant negative version of the PP2A catalytic subunit (*mts^{DN}* flies). Therefore, the majority of PP2A functions on CLK are mediated by the CKA regulatory subunit (Fig.5). CKA coimmunoprecipitated with CLK in S2 cells, supporting the existence of CKA/CLK complexes.

PP2A holoenzymes containing a regulatory subunit other than CKA also exist. RNAi-mediated downregulation of the WDB B''-type PP2A regulatory subunit also demonstrated an alteration of the CLK protein. Head extracts of *wdb*-RNAi expressing flies showed reduced CLK protein quantities while it did not affect (gross) CLK phosphorylation. This was in agreement with previous data from cultured S2 cells showing a strong CLK destabilization after treatment with dsRNAs targeting *wdb* (Kim and Edery, 2006). Altogether, we could describe two alternative PP2A complexes that differentially regulate the CLK protein: PP2A/CKA controls CLK de-phosphorylation during daytime, while PP2A/WDB adjusts global CLK quantities throughout the cycle (Fig. 5).

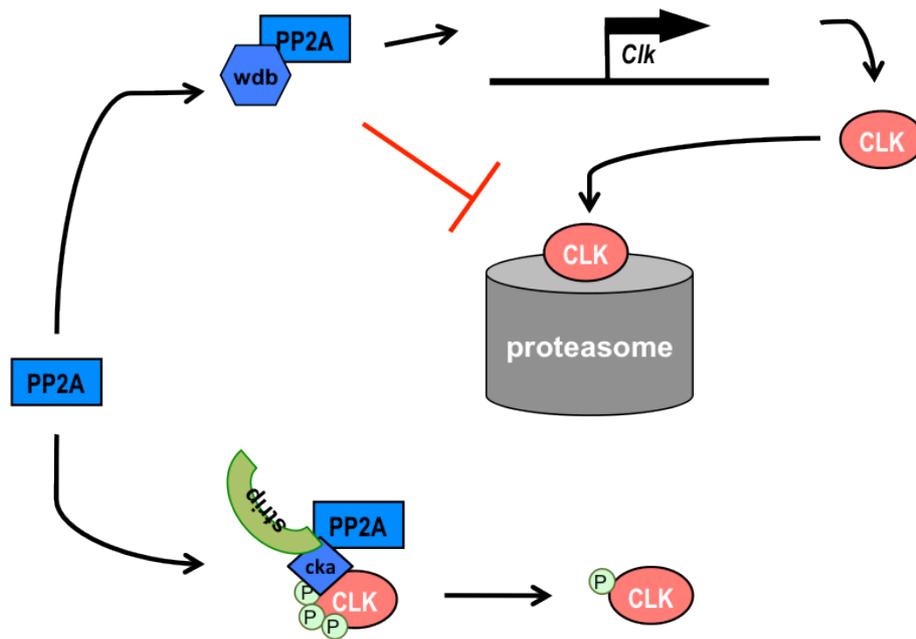


Fig. 5 Two alternative PP2A complexes differently regulate the CLK protein

The association of a defined regulatory subunit to the core catalytic dimer determines the substrate specificity and localization of the PP2A holoenzyme. Both Cka and Wdb regulatory subunits affect the CLK protein. PP2A/Cka controls CLK dephosphorylation and reactivation, while PP2A/Wdb affects the overall CLK quantities by regulating *Clk* gene transcription, CLK stability, or both.

b. PP2A/Cka functions affect CLK transcriptional ability (more than PP2A/Wdb)

CLK transcriptional ability correlates with its phosphorylation status. Hypo-phosphorylated CLK is present at times of maximal transcription of CLK target genes, while hyper-phosphorylated CLK is associated with transcriptional silencing (Yu et al., 2006).

Given the long-period phenotype, we expected to see a delay in the transcription of CLK targeted genes. Yet, quantitative-PCR data at the second day of constant darkness showed that the phase of *vri* and *tim* gene transcription was not changed either by *Cka* or *wdb* (or *strip*) downregulation. However, *Cka*-RNAi severely reduced both *tim* and *vri* gene transcription. Hence, PP2A/Cka activities are important for the transcription activation functions of CLK. On the other hand, *wdb* downregulation had either a limited (*tim* pre-mRNA) or no impact (*vri* mRNA) on CLK transcriptional activity. Therefore, the degree of phosphorylation of CLK (*Cka*-RNAi) appears much more important than its quantity (*wdb*-RNAi) for proper CLK-mediated gene transcription. Further support to this idea comes from a mutation in the

CK2 α kinase ($CK2\alpha^{Tik}$) that causes very low non-oscillating levels of CLK, yet supports high transcription from CLK-controlled promoters (Szabo et al., 2013). Hypo-phosphorylated, transcriptionally active CLK during the daytime is supplied either by the translation of *Clk* mRNAs (that peak in the early morning) or by the de-phosphorylation of the hyper-phosphorylated CLK that has left the chromatin at the end of the cycle (Hardin, 2011). We suggest that the PP2A/Cka regulate this latter pathway (Fig. 6).

Cka was already shown to associate with transcription factors and regulate their activity. Cka was first isolated as a multi-domain protein organizing a functional kinase and transcription factor-containing complex that turned on AP-1 -regulated genes (Chen et al., 2002). *Kayak* (*kay/Dfos*) is the *Drosophila* homolog of the mammalian *c-fos* gene that, together with *c-jun* (*Djun/jra* in *Drosophila*), forms the AP-1 transcription complex. Interestingly, *kayak* was among the putative new *Drosophila* clock genes isolated in our screen. Moreover, the KAY- α protein was shown to act within the core clock to control both amounts and activity of the CLK protein (Ling et al., 2012).

Several lines of evidence suggest that the Cka activity on CLK may involve the KAY- α protein. First, Cka coimmunoprecipitated with Kay in embryonic lysates as well as in cultured cells (Chen et al., 2002). Second, expression of either *Kay- α* - or *Cka*-RNAi in Pdf⁺ neurons lengthened the period of locomotor activity of flies by approximately the same extent (+1,5h). Third, both *Cka* and *kay* downregulation affected transcription of CLK target genes. Intriguingly, our qPCR results showed a variable “penetrance” of *Cka*- and *strip*- (or *Wdb*-) RNAi effects on the downregulation of *tim* and *vri* pre-mRNAs curves, *tim* pre-mRNA being much more affected. Similarly, TIM, PER, PDP1 and VRI quantities in s-LNvs were variably modified by *kay- α* downregulation.

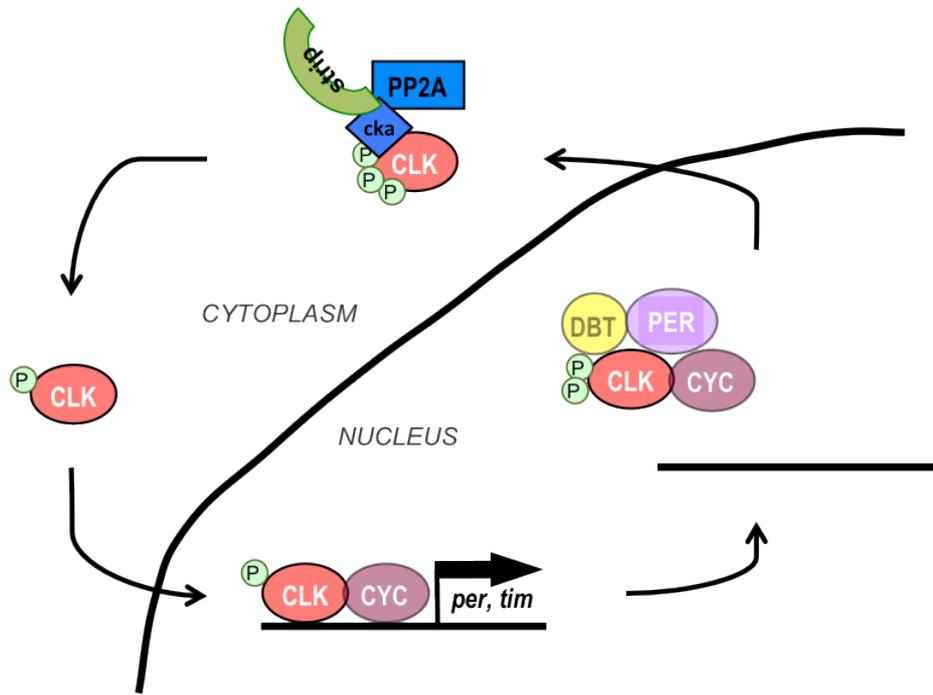


Fig. 6 The PP2A/Cka/Strip complex controls CLK reactivation

Helped by the Strip anchoring, PP2A/Cka contacts the hyper-phosphorylated CLK leaving the nucleus after transcriptional repression, and dephosphorylates it. CLK dephosphorylation allows its reactivation. Hypo-phosphorylated CLK can re-enter the nucleus and drive another cycle of transcription.

c. Strip cooperates to mediate PP2A/Cka functions on CLK, possibly controlling localization of the STRIPAK complex

Regulatory subunits are known to bridge PP2A and compartment-specific adaptors to place the holoenzyme in vicinity to its targets (Janssens and Goris, 2001; Herzog et al., 2012). Cka strongly associated with the Strip protein in fly head extracts as well as in cultured S2 cells. Moreover, *strip* downregulation recapitulated the hyper-phosphorylated CLK phenotype of *Cka*-RNAi expressing flies and no additional modifications were shown on CLK when RNAi expression for both genes accomplished simultaneously. Hence, we conclude that Strip does collaborate with Cka to coordinate PP2A/Cka functions on CLK (Fig. 6).

The biological roles of *Drosophila* Strip, as well as those of its homologs in other species are, to date, not known. Yet, the Strip polypeptide contains several conserved hydrophobic motifs that were proposed to promote the localization of the yeast Strip protein within the Golgi membrane, in vicinity to the nuclear envelope (Frost et al., 2012). Therefore, Strip-containing complexes may participate in the regulation of proteins entering/leaving the nucleus. Fractionation studies on *strip*-RNAi extracts indicated that less (and more

phosphorylated) CLK was present in the nucleus at times of maximum CLK activity (ZT15), in agreement with reduced *tim* and *vri* transcripts. CLK staining was indeed higher in the cytoplasmic fraction of *strip* downregulated flies compared to controls. Data supported a role for the Strip-containing PP2A/Cka complex in regulating CLK cytoplasm-nuclear shuttling: PP2A/Cka dephosphorylating activities promote CLK nuclear localization (Fig. 6). A similar role is played by PP2A in the *Neurospora* circadian system: PP2A-mediated WCC dephosphorylation allows the entry of the transcription factor complex into the nucleus (Tataroglu and Schafmeier, 2010).

Experiments done in *Drosophila* and human cultured cells showed that *strip* downregulation affected the cell cytoskeleton organization of actin and tubulin filaments (Bai et al., 2011). In addition, Strip associates with the motor protein dynein (Hutchins et al., 2010). All those data indicate that the Strip protein localizes in the cytoplasm, where it mediates anchoring of the STRIPAK complex.

d. Strip has some Cka-independent functions

Both *Cka* and *strip* downregulation altered the phosphorylation profile of the CLK protein. In addition, *strip*-RNAi expression induced a stabilization of the PER and TIM proteins, which was not seen in *Cka*-RNAi extracts. Hence, Strip had some Cka-independent functions. PER was stabilized by *strip*-RNAi expression also in a *tim*⁰ background, suggesting that Strip took part in the destabilization of the PER protein in a TIM-independent manner. As PER is essentially cytoplasmic in a *tim* null background, Strip-mediated changes on PER must occur in the cytoplasm. Higher PER staining after *strip*-RNAi expression was also shown in a *dbt*^{K/R} kinase-dead background. This further lends credence to the idea that Strip regulates the breakdown of non-Dbt-phosphorylated PER forms (Fig. 7).

These Strip functions could be unrelated to PP2A; alternatively, another PP2A regulatory subunit could mediate Strip activities on PER (Fig. 7). Analysis of Strip immunocomplexes of fly head extracts showed that the Cka protein interacted with Strip at all time points. Remarkably, the large majority of Cka was pulled-down with Strip (indeed, very low Cka was observed in supernatant extracts), while neither Wdb nor Tws PP2A regulatory subunits were found in Strip immunocomplexes. The Strip binding to a PP2A regulatory subunit (at least among the regulatory subunits tested) seemed thus exclusive to Cka. However, Strip-containing complexes including a PP2A regulatory subunit different from Cka were proposed

to exist in *S. cerevisiae*, where Far11 (Strip homolog) was reported to interact with a B'-type PP2A regulatory subunit (Pracheil et al., 2012).

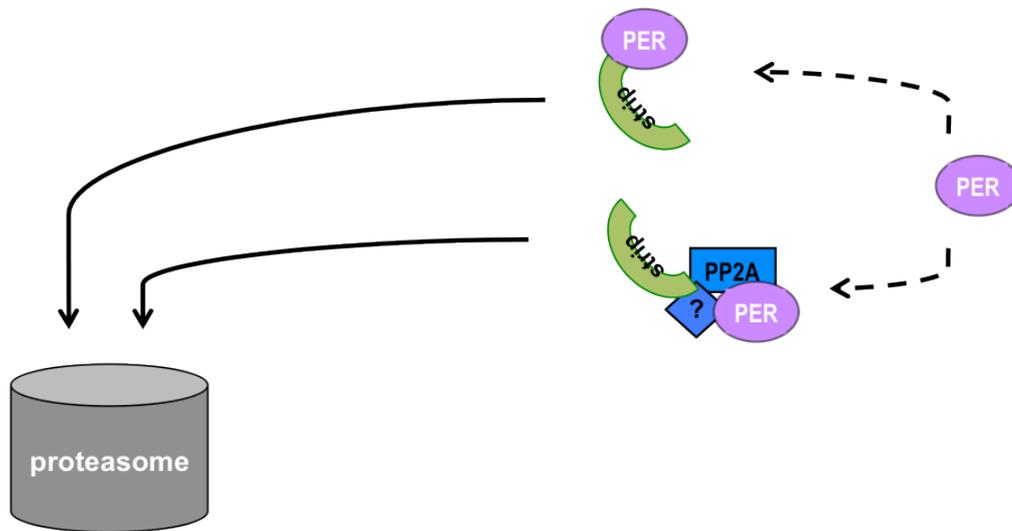


Fig. 7 Strip destabilizes non-TIM-bound PER independently of Cka

Strip participates to the destabilization of the PER protein that is not bound to TIM. In addition, Strip controls the breakdown of non-Dbt-phosphorylated PER. This role of Strip is independent of Cka but may involve a PP2A complex containing a different regulatory subunit. Alternatively, Strip favors PER degradation directly or by bridging PER with the protein targeting its breakdown.

e. Functional connections between known CLK kinases and the PP2A/Cka phosphatase activities

Little is known about the kinase(s) affecting CLK phosphorylation. In addition to Doubletime (Dbt), the CK2 and Nemo (Nmo) kinases were shown to affect CLK phosphorylation and quantity (Yu et al., 2011; Szabo et al., 2013).

To test the relationship between Dbt and PP2A, we used overexpression of a kinase-dead Dbt polypeptide ($dbt^{K/R}$ flies) (Muskus et al., 2007) together with a heterozygous hypomorphic *dbt* mutation (dbt^{ar}). The medium-phosphorylated CLK profile shown by $dbt^{K/R}, dbt^{ar}$ flies was not altered by RNAi-mediated downregulation either of Strip or of Cka, suggesting that Dbt worked upstream of PP2A/Cka to regulate CLK. In addition, we showed that the large majority of Cka-RNAi effects on CLK hyper-phosphorylation were abrogated in a heteroallelic *nmo* mutant background (nmo^{P1}/nmo^{Df}). We conclude that both Dbt and Nemo phosphorylation activities are required for CLK to be a suitable substrate for PP2A/Cka.

In contrast to Nemo and Dbt, deregulation of the CK2 α kinase functions (*CK2 α ^{Tik}* overexpression) did not prevent the CLK hyper-phosphorylation effects of Cka downregulation. Indeed, more hyper-phosphorylated CLK forms accumulated in *tim>Cka-RNAi,CK2 α ^{Tik}* flies. Therefore, CK2 α and PP2A/Cka affect the CLK protein in a completely independent and additive manner.

f. The Drosophila STRIPAK complex contains members of the Ste-20 family kinase (that can be a substrate for PP2A activity)

strip and Cka, together with PP2A catalytic and scaffold subunits are part of an extremely well conserved super-complex called Striatin-interacting phosphatase and kinase complex (STRIPAK). In addition, members of the *Drosophila* Ste-20 kinase family, such as *misshapen (msn)* and *germinal center kinase III (gck-III)*, co-purified with the major STRIPAK components in *Drosophila* cultured cells (Ribeiro et al., 2010).

We showed that the RNAi-mediated downregulation of either *msn* or *gck-III* in clock cells affected the fruit fly locomotor rhythms, indicating that the activity of these two kinases may modulate the pace of the *Drosophila* molecular clock. A recent study showed that PP2A dephosphorylation inhibits Misshapen-like kinase (MINK) activity in humans (Hyodo et al., 2012). In addition, PP2A/Cka controls dephosphorylation of the *Drosophila* Hippo (Hpo) kinase, which is another member of the Ste-20 family kinase (Ribeiro et al., 2010). These evidences suggest an alternative way to explain the hyper-phosphorylated CLK phenotypes of *Cka*-RNAi expressing flies: the STRIPAK complex may control CLK phosphorylation through regulating the activity of a (CLK-) kinase in the complex. Ste-20 family kinases are known to auto-phosphorylate at the catalytic site to increase their activity (Hata et al., 2013). Therefore, activated PP2A/Cka within the STRIPAK complex can control kinase dephosphorylation and inactivation. In this scenario, low PP2A phosphatase function (*Cka*- or *strip*-RNAi) allows auto-phosphorylation and activation of the Ste-20 family kinase in the complex that will in turn hyper-phosphorylate CLK. Within the STRIPAK complex, kinases and phosphatases can reciprocally regulate each other's activity. Whether CLK is a substrate for a kinase of the complex – and PP2A/Cka regulates kinase activity, or phosphorylated CLK is a direct target for PP2A/Cka-mediated dephosphorylation remains to be investigated.

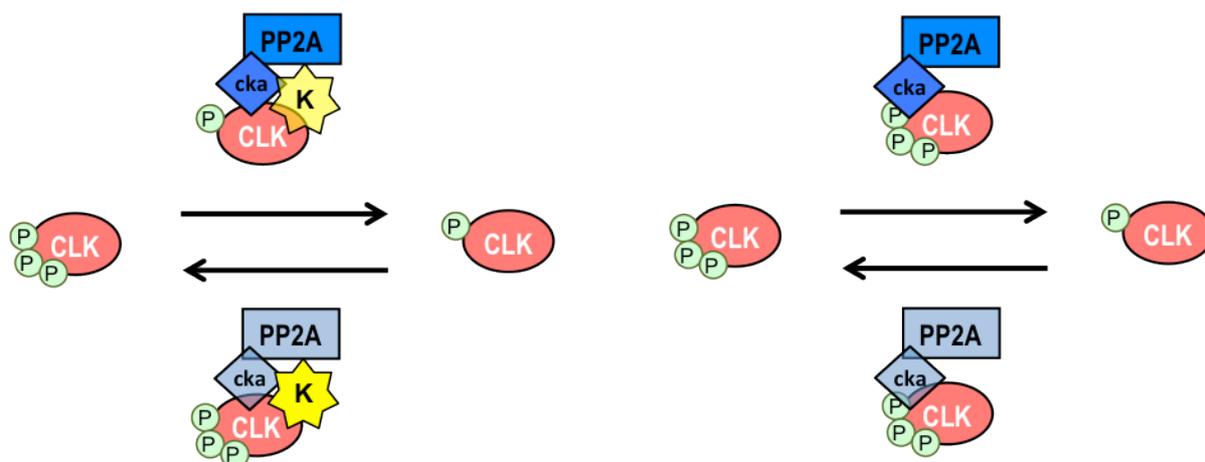


Fig. 8 The STRIPAK complex can affect CLK phosphorylation by two alternative mechanism

Within the STRIPAK complex, kinase and phosphatase enzymes coexist and reciprocally regulate each other's activity. The STRIPAK complex may directly regulate CLK dephosphorylation by PP2A/Cka binding to hyper-phosphorylated CLK (right). PP2A/Cka can also control dephosphorylation and inactivation of a STRIPAK-included kinase that phosphorylates CLK (left). Intriguingly, both mechanisms can cooperate to produce a stronger response.

g. The role of PP2A complexes in the Drosophila molecular clock

Data presented here gave evidence of PP2A regulating the phosphorylation status of the CLK protein. We propose a role for PP2A/Cka in the dephosphorylation of the hyper-phosphorylated CLK that accumulates as an inactive soluble protein at the end of the cycle (in the morning). Highly phosphorylated CLK is thought to leave the nucleus and join the cytoplasm for further phosphorylation and (possibly) degradation (Hung et al., 2009). In our model, thanks to Strip-mediated anchoring, PP2A/Cka localizes in proximity to the nuclear envelope where it contacts hyper-phosphorylated CLK leaving the nucleus. PP2A/Cka-mediated dephosphorylation reactivates CLK and, possibly, it promotes its nuclear re-entry. Therefore, PP2A/Cka activity reinforces the pool of hypo-phosphorylated CLK available for a novel cycle of transcription.

In contrast to PP2A/Cka, PP2A/Wdb complexes control the global amount of clock components. Indeed, not only CLK but also PER and TIM protein levels are severely reduced after *wdb*-RNAi expression. In contrast, *wdb* overexpression increases PER levels (Sathyanarayanan et al., 2004). Unlike Cka, Wdb depletion dampens transcription of the *Clk*

gene. Reduced CLK quantities give rise to reduced transcription of clock-controlled genes, which weakens the high amplitude oscillations of core clock genes as well as output genes regulating rhythmic behaviors.

III. SUMMARY OF THE OBTAINED DATA

The key positive and negative components of the molecular feedback loop(s) underlying *Drosophila* circadian rhythms have been established. Nevertheless, additional partners are required to describe the fine-tuning of the loop and the pathways by which the clock may sense the external entraining signals and synchronize.

A large-scale behavioral screen based on targeted RNAi-mediated gene knockdown has allowed the isolation of 54 putative new clock genes (RESULTS §I). In this work, three putative new clock genes identified during the screen have been analyzed for their functions in the phase-setting of the molecular clock of *Drosophila* (Fig. 9).

Expression of the *CG17350*-RNAi transgene in clock cells produces long-period but strongly rhythmic behaviors (see ANNEXES §III). Molecular analysis shows a PER-dependent stabilization of hypo-phosphorylated TIM during the morning, suggesting a defect in the mechanisms targeting TIM phosphorylation. However, analysis of the PER protein oscillations in the larval clock neurons of *CG17350* null mutants shows no apparent defects, indicating that effects of the RNAi are likely to be the consequence of the downregulation of a gene other than *CG17350*. Therefore, the analysis on *CG17350* has not been continued further. This represents an example of the limits of the RNAi technique for gene silencing.

Another gene analysed is the Usp5 deubiquitinylase enzyme (RESULTS §I). Downregulation of Usp5 in clock cells lengthens the period of locomotor activity of flies as well as PER protein oscillations in clock neurons. High molecular weight forms of PER and TIM proteins accumulate during the morning after *Usp5* knockdown, but TIM was not stabilized in the absence of PER, whereas PER is still stabilized in a *tim⁰* background. Therefore, Usp5 directly participates in the degradation of the PERIOD (PER) protein and, later, of the TIMELESS (TIM) protein at the end of the cycle. In addition, Usp5 also controls the stability of the CLOCK (CLK) protein. Being an ubiquitin protease, Usp5 may directly deubiquitinate PER. However, according to the role described for the homolog protein in humans, Usp5 may control PER degradation more indirectly. Indeed, Usp5 disassembles the unanchored polyubiquitin chains naturally accumulating in the cell; these could compete with ubiquitinated-PER for proteasome recognition and thereby blocking PER breakdown.

The large majority of my studies have been conducted on a third putative clock gene, *CG11526/Strip* (RESULTS §II). *Strip* interacts with Connector of Kinase to AP-1 (*Cka*), a novel regulatory subunit for the PP2A phosphatase holoenzyme, both in insect S2 cells and in fly head extracts. Downregulation of both *Strip* and/or *Cka* causes long-period behavioral phenotypes and high molecular weight forms of the CLOCK (CLK) protein to accumulate in the morning. Perturbation of general PP2A activity also produces hyper-phosphorylated CLK in the morning suggesting that *Cka/Strip* mediates CLK dephosphorylation by PP2A at the end of the cycle. Hyper-phosphorylated CLK is transcriptionally inactive. Indeed, transcription of CLK-target genes is strongly reduced in *Cka*-RNAi flies. PP2A has already been shown to affect CLK stability in insect S2 cells (Kim and Edery, 2006), and these effects are mediated by the *Widerborst* (*Wdb*) or *Twins* (*Tws*) PP2A regulatory subunits. We show that *Wdb* downregulation also affects the stability of CLK in fly head extracts, but has no apparent effects on CLK phosphorylation. Therefore, at least two different PP2A complexes can be described that modify the CLK protein: the PP2A/*Cka/Strip* complex controls CLK dephosphorylation and reactivation, whereas PP2A/*Wdb* affects CLK stability independently and/or after PP2A/*Cka* functions. In addition, PP2A/*Wdb* controls global amounts of the PER and TIM proteins. Moreover, *Strip*, but not *Cka*, destabilizes PER independently of TIM and PER phosphorylation by the *Dbt* kinase. This indicates that *Strip* possesses some functions unconnected to *Cka*. Finally, the *Cka*-containing PP2A and *Strip* are part of a large supercomplex called STRIPAK (striatin interacting kinase and phosphatase). In addition to the PP2A phosphatase, this complex contacts members of the germinal center kinase of the Ste-20 group. Within this complex, the phosphatase and kinase are likely to reciprocally regulate each other activities. Therefore, in addition to directly dephosphorylate hyper-phosphorylated CLK, the STRIPAK complex may also favor CLK hypo-phosphorylation through the (PP2A-dependent) inhibition of a CLK kinase included in the complex.

In conclusion, the screen performed has allowed the isolation of (at least) two new components of the *Drosophila melanogaster* molecular clock. The analysis on other positive genes is in progress in the laboratory.

We have evidence from the literature about a number of ubiquitin-ligase enzymes (*Slmb*, *Jetlag*, *Cul-3*, *CTrip*) that modify the major clock proteins. Here, we give evidence for the circadian role for an ubiquitin protease enzyme, *Usp5*. Together with *Usp8* functions on CLK deubiquitylation, *Usp5* represents one of the first ubiquitin-protease enzymes isolated to function within the circadian clock of the fruit fly.

Studying the circadian role of the Strip protein (isolated in the screen) we have provided important informations concerning the post-translational control of the CLK protein. Indeed, very little is known about the mechanisms that regulate CLK stability and transcriptional activity. Our study proves a double role for the PP2A phosphatase in the control of the CLK protein in adult flies. Importantly, the STRIPAK complex, containing both Strip and the PP2A/Cka proteins, is essential for the generation of the hypo-phosphorylated CLK forms that drive transcription of clock-controlled genes. In addition, a PP2A/Wdb complex is implicated in the control of the overall amounts of the CLK protein.

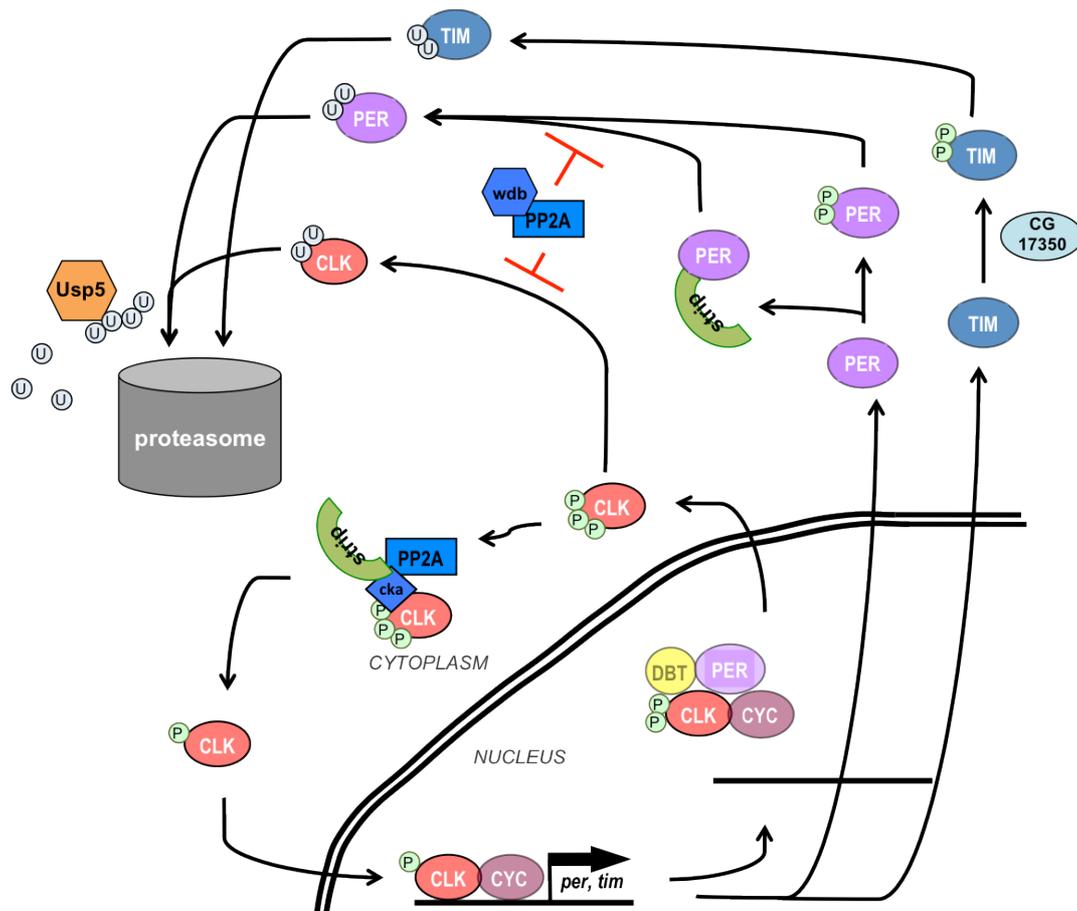


Fig. 9 Summary scheme

The protein targeted by expression of the *CG17350*-RNAi (here illustrated as CG17350) affects TIM phosphorylation and stability in a PER-dependent manner. Conversely, the Usp5 deubiquitinylase controls degradation of the PER protein, possibly through the disassembly of poly-ubiquitin chains that, if accumulating, compete with ubiquitinated PER protein for proteasome binding. Finally, the Strip protein (or a Strip-linked protein) participates in the degradation of PER, while a Strip-containing PP2A/Cka complex mediates dephosphorylation and re-activation of the CLK transcription factor. In addition, a Wdb-based PP2A holoenzyme sets the overall quantities of all major circadian proteins (CLK, PER, TIM).

ANNEXES

I. HOW TO ASSESS *Drosophila* LOCOMOTOR RHYTHMS

Drosophila locomotor rhythms are assessed as follows (Fig.1) (Rosato and Kyriacou, 2006). Each fly is inserted in a single glass tube, half filled with sucrose-containing gelatin, and loaded in 32-channel activity monitors, which are housed in temperature- and light-controlled incubators. Each channel in the monitor is equipped with infrared emitters and receivers, associated with a computer: every time the fly move through the detector, the IR beam is break and a signal is sent to the computer. Motion data are assembled each 30 minutes (bin), and recording of each bin up to 24h indicate the daily pattern of activity and rest for each fly.

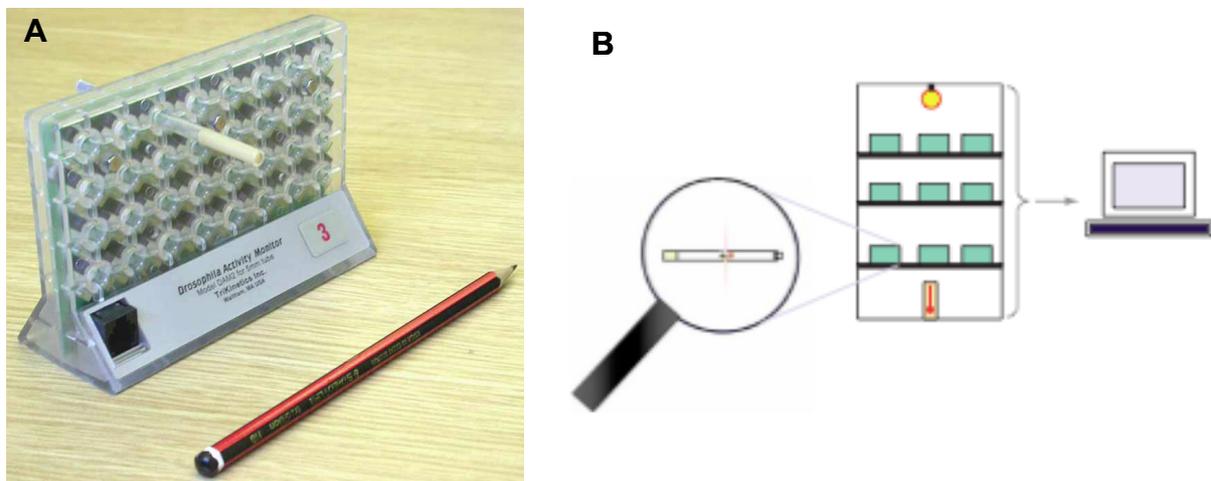


Figure 1 Setup for the analysis of *Drosophila* locomotor rhythms.

A. Image of *Drosophila* activity monitors (from TriKinetics). **B.** Setup for locomotor activity analysis. Individual flies are inserted in small glass tubes and loaded in activity monitors (green boxes). Monitor are housed in a light- and temperature-controlled incubator and connected to a computer. The activity monitors contain the infrared emitters and receivers and the associated computer components. (Rosato and Kyriacou, 2006)

Raw data can be represented as *actogram* or *eductions* (see § II.3). Actograms show locomotor behaviors during multiple days and provides informations about the period and phase of the internal clock. Eductiones give readout of the average locomotor activities during a 24h time-scale and are used to assess anticipatory behaviors in light-dark (LD) conditions.

Raw data about the peak recurrence under free-running conditions are treated statistically to obtain informations about the period of the oscillating behavior. In our laboratory we use a

χ^2 periodogram analysis. The periodogram (Fig. 2) is a graph estimating the power of the rhythm as a function of the tested period. Among the parameters calculated by this statistical analysis are the period of rhythmic flies (the period that corresponds to the highest power of the periodogram) and its associated power, which reflects the robustness of the rhythm. The power value is given as the height of the periodogram peak above the 1 or 5% significance threshold, which is arbitrarily chosen for the χ^2 analysis. Other parameters of the periodogram are the width and the complexity of the peak, as well as the total number of significant peaks that are detected. Multiple peaks indicate that several rhythmic components are detected giving rise to more complex rhythmicity.

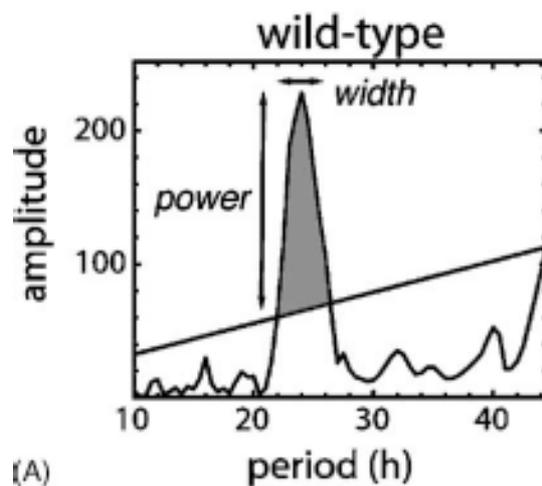


Figure 2 Representative periodogram derived from activity records of individual flies in constant darkness. The periodogram correspond to at least 5 days of activity, beginning 24 h after the last light-OFF transition. Power of the rhythm is defined as the height of the main peak above the line corresponding to the confidence limit (here a 95% confidence limit was used). The width of a peak is also defined relative to that line (Klarsfeld et al., 2003).

II. THE GAL4-UAS BINARY SYSTEM AND THE RNAi TECHNIQUE

1. The Gal4-UAS binary system

The Gal4-UAS binary system is a key tool widely used by researchers to target gene expression in specific tissues or in a set of cells. Identified in the yeast *S. cerevisiae*, the *Gal4* gene encodes for a 881-amino acids protein that shows transcription factor properties. Gal4 binds sites named *Upstream Activating Sequences (UAS)* and drive transcription of the downstream sequence.

Taking advantage of the existence of P-transposable elements in *Drosophila*, Brand and Perrimon have developed the Gal4-UAS system to force expression of exogenous genes of interest in this model organism (Brand and Perrimon, 1993). Two different lines are employed:

- Driver “Gal4-line”. The *Gal4* gene, under control of a weak promoter, is randomly inserted in the fly genome through the flanking region of a P-element; this construct will drive *Gal4* expression from the different genomic enhancers around the insertion site. Alternatively, specific regulatory sequences (promoter) can be sub-cloned upstream of the *Gal4* gene in a cloned vector which is then randomly inserted in the genome; this construct will drive *Gal4* expression in a tissue/cells-specific manner (accordingly to the promoter sequences used).
- Responder “UAS-line”. The Gal4-dependent targeted gene (gene *X*) is sub-cloned downstream of *UAS* sequences in a transposable P-element, which is randomly inserted in the fly genome.

The absence of *Gal4* in the UAS-line maintains the target gene in a transcriptionally silent state. To activate transcription, flies carrying the *UAS-geneX* sequence are crossed with flies expressing the *Gal4* gene: the outcome progeny will express the gene of interest (*X*) with a transcriptional pattern that reflects the pattern of the *Gal4*-driver.

2. Gal4-UAS approach to RNAi

RNA interference (RNAi) is a post-transcriptional, highly conserved process in eukaryotes that leads to specific gene silencing through degradation of the target mRNA. This

mechanism is mediated by the formation of a double-stranded RNA (dsRNA) that is homolog in sequence to the silenced gene. The dsRNA is processed into small interfering RNA (siRNA) by an enzyme called Dicer, and the siRNAs (19-23 bp fragments) are then incorporated into a multi-component RNA-induced silencing complex (RISC), which finds and cleaves the target mRNA.

Flies encoding a *Gal4* driver are crossed to flies encoding *UAS-RNAi* targeting a specific *Drosophila* gene (Fig.3). In the resulting offspring, the Gal4 driver binds to UAS sequences and drives the expression of inverted repeats in a cell-specific manner (Gal4 promoter/enhancer). Hairpin RNAs (hpRNAs) that results from the expression of the inverted repeat are processed by *Dicer2* (*dcr2*) into 21- to 23-nucleotide segments (siRNA), which are then incorporated into the RISC complex for recognition and degradation of endogenous mRNA transcripts (Carthew, 2001); (Liu and Paroo, 2010).

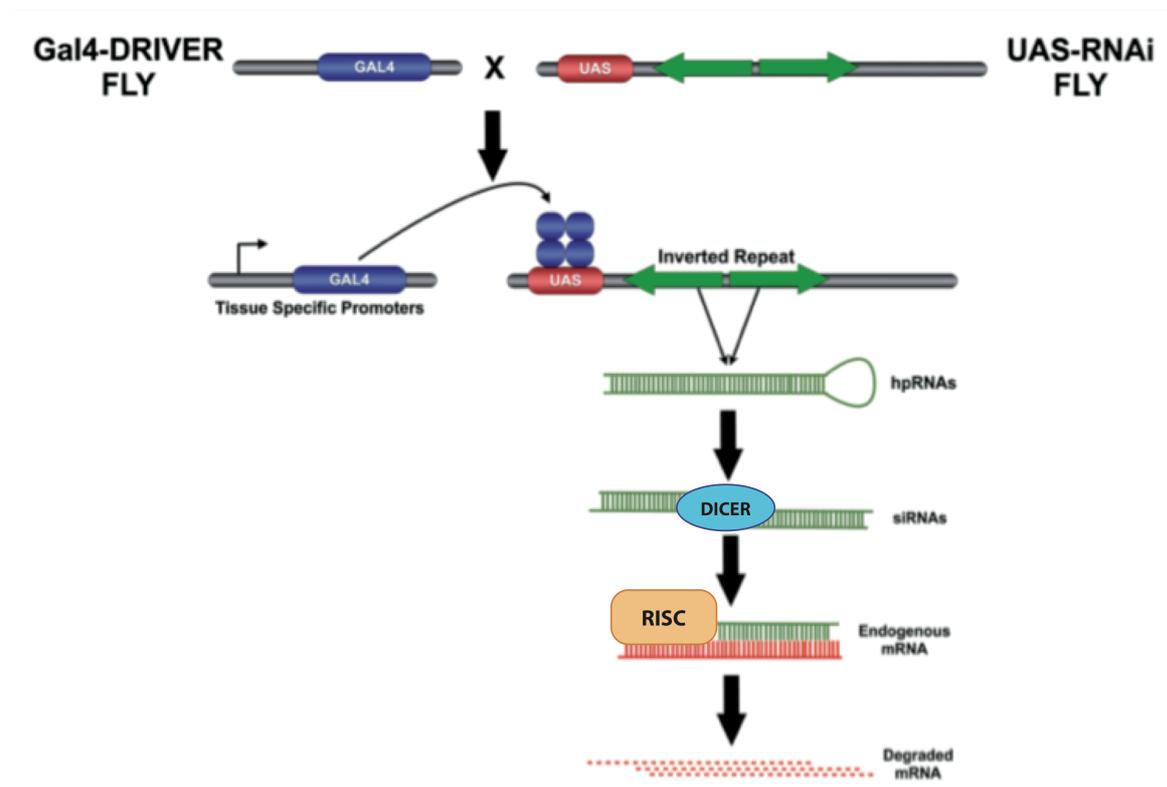


Figure 3 Gal4-UAS approach to RNAi

The Gal4-UAS system allows targeted expression of a double-stranded mRNA (dsRNA) of 350-500 bp in a specific cell or tissue. dsRNA is processed by Dicer, that cuts the strand in 19-23 bp fragments. The small fragments of dsRNA are recognized by the RISC protein complex, which degrades one of the two strand of the fragment and uses the other to match with the endogenous mRNA. Then the RISC complex destroys the endogenous mRNA. Schema adapted from a similar diagram posted at the VDRC website. <http://stockcenter.vdrc.at/control/rnailibrary>.

III. Unpublished data: CG17350-RNAI EXPRESSION IN CLOCK CELLS LENGTHENS THE PERIOD OF THE LOCOMOTOR BEHAVIOR OF FLIES

1. Results

a. CG17350 downregulation lengthens the period of the fly locomotor behaviors

We isolated the *CG17350* gene in the RNAi screen.

A NIG-FLY RNAi pairs to the *CG17350* mRNA in the second and third coding exons (two independent insertion lines are available, named R1 and R2). Two additional transgenic fly lines, bearing largely overlapping RNAi sequences targeting the fourth and last exon, were obtained from the VDRC database (fig.1A). Flies expressing the *17350-R2* RNAi line from NIG-FLY (hereafter referred as *CG17350*-RNAi) either in the s-LN_v neurons or, more broadly, in all TIM-positive cells showed a robust 3 hours-lengthening of the circadian period of the fly locomotor behavior, while remaining strongly rhythmic (Table1). In contrast, expression of either of the two VDRC RNAi lines and also of the R1 RNAi line from the NIG-FLY database showed no altered behavioral phenotype.

Transposable elements can insert within a gene span and affect its transcription. Two independent fly lines from the Bloomington stock center possess a transposon inserted within the *CG17350* gene. The #18182 line has a PBac insertion in the first intron and the #23195 line contains a Minos element insertion at the non-coding 3' end of the gene (Fig.1A). In contrast to PBac elements, Minos elements can excise imprecisely and can be used to generate genomic deletions. Excision of the Mi(ET1)MB0546 element was induced in a genetic background favoring non-precise excisions (*mus309^{NI}/mus309^{D2}* heteroallelic mutant background; (Witsell et al., 2009)). More than one hundred excised lines that had lost the red-eye colored *mini-white* marker of the transposon were obtained. Homozygous viable flies were behaviorally tested, but none of them showed altered locomotor rhythms. Eleven excised lines were homozygous lethal. PCR analysis revealed that the *fondue* neighbor gene was deleted in the eleven lines, whereas *CG17350* was deleted in seven of them. We concluded that the deletion of the *fondue* gene was lethal, but we could not conclude for *CG17350*. Two different RNAi lines exist that have been shown to efficiently downregulate *fondue*

expression. *fon*-RNAi expressing flies were behaviorally tested and showed no altered locomotor behaviors (not shown).

A collaboration was started with Dr. Dushay in Chicago (IL), who is working on the *fondue* gene. The *fondue* gene is involved in hemolymph clotting and coagulation processes. We were provided with a UAS-*fon* line to rescue *fon* and *fon/CG17350* double mutants. Broad expression of UAS-*fon* using a *daughterless(da)*-GAL4 driver rescued the lethal phenotype of the *fondue/CG17350* mutant background ((Bajzek et al., 2012)– see ANNEXES § IV), suggesting that *CG17350* is not essential for adult development. However, survival rates were low in our hands (possibly because the expression of UAS-*fon* in DA-positive cells, only partially coincides with wild-type *fon* expression). We thus could not obtain a sufficient number of healthy flies to generate significant behavioral results.

b. Altered PER and TIM protein oscillations in sLN_vs of CG17350-depleted adult flies.

To analyze the molecular consequences of *CG17350* depletion, we looked at PER and TIM oscillations in the s-LN_v of flies expressing the *CG17350*-RNAi in *tim*-positive cells (Fig.1B). TIM levels still cycled although the oscillations were slowed down and had reduced amplitude. Much stronger effects were observed for PER, with constitutively very low levels of detected protein.

Fon null mutants (as well as *fon/CG17350* double mutants) survive until the 3rd instar larval stage and die before pupariation. A double *fon/CG17350* mutant line ($\Delta 22$) that was fully deleted for the *CG17350* gene but only partially deleted for the *fon* gene (C-terminal part missing) was chosen to assess the oscillation profile of PER and TIM proteins in the larval sLN_vs (a *fon*-only deletion, $\Delta 17A$, was used as a control). Preliminary data (Fig.1C) suggested that both PER and TIM proteins oscillated in *CG17350/fon* double mutants with a phase and amplitude similar to *fon* single mutants – similarly to wild type flies. The data thus indicated that the loss of *CG17350* had no impact on the clock protein oscillations in the larval brain. This was rather different from the severely affected oscillations and quantity of the PER protein in adult flies expressing *CG17350*-RNAi. Results in larva (mutants) and adults (RNAi) were thus inconsistent. The possibility rised that the altered behavioral effect induced by *CG17350*-RNAi expression was due to the downregulation of an off-target gene and not *CG17350*.

c. *CG17350*-RNAi expression induces TIM stabilization in the subjective morning

The molecular effects of *CG17350* depletion were analyzed by Western blots. Head extracts of flies expressing *CG17350*-RNAi in *tim*-positive cells (*tim-gal4/+;UAS-dcr2/UAS-CG17350-RNAi*) were compared to control extracts (*tim-Gal4/+;UAS-dcr2/+*). Flies were collected every 3 hours during an LD cycle and on the first day of constant conditions (DD). As shown in Fig.2A, molecular oscillations at the PERIOD protein were not affected by *CG17350*-RNAi expression. The CLK protein showed lower quantities in the morning, both in LD and DD. Stronger effects were observed for the TIM protein, which was stabilized during the subjective day in DD. Such effects were not seen in LD conditions, likely because of the “masking” effect of the light-dependent TIM breakdown. The TIM forms that accumulated in *CG17350*-RNAi extracts were rather low-phosphorylated forms. This suggested a role for *CG17350* in promoting the TIM phosphorylation events that are involved in the circadianly-controlled “morning” TIM degradation program.

To test if the RNAi expression directly affected the TIM protein we used a *per* null mutant background (*per*⁰). No TIM stabilization was observed in *per*⁰; *tim-GAL4/+; UAS-dcr2/UAS-CG17350-RNAi* head extracts (Fig.2B). This indicated that the PER protein was required to observe the stabilizing effects of *CG17350*-RNAi expression on TIM. In a *per*⁰ background, the TIM protein that accumulates is hypo-phosphorylated (Zeng et al., 1996) and localizes in the cytoplasm (Myers et al., 1996). This means that if the action of *CG17350* on TIM takes place in the nucleus we cannot expect to see an effect in a *per*⁰ background, when TIM is essentially cytoplasmic. Indeed, at times when we see TIM stabilization in RNAi flies (i.e. during the morning) the TIM protein is expected to be essentially nuclear.

2. Figure Legends:

Table1 Locomotor activity of flies expressing CG17350-RNAi.

Flies were kept at 25°C and exposed to at least 2 days of 12:12 LD followed by 11 days of DD conditions. Rhythmicity is calculated using the last 10 days of experiment. Results are referred to male flies. #17350-R2: *CG17350*-IR insertion line from the NIG-FLY stock center. VDRC #30753 and VDRC #106106 insertions from VDRC recognize partially overlapping sequences on *CG17350* mRNA. #18182: PBac insertion from the Bloomington stock center; #23195: Minos element insertion from Kyoto.

^aTotal number of flies that survived until the end of the testing period

^bPercentage of flies with activity rhythms having a power value of ≥ 30

^cMeasure of the strength or amplitude of the rhythm.

Fig. 1 The *CG17350* gene and behavioral effects of *CG17350* depletion

A. Transcription from the *CG17350* locus gives rise to one unique mature mRNA. Position of the targeted sequences from the VDRC (KK#110762 and GD#5329) and NIG-FLY (17350-R2) RNAi databases are shown. Location of the transposable element insertions is also shown. **B.** PERIOD and TIMELESS oscillations in the s-LN_vs of adult flies expressing *CG17350*-RNAi are perturbed. Flies were entrained to 12h light-12h dark conditions (LD) for four days and then collected every 3 hours during the last day of LD and the first day of constant darkness. Around ten flies were dissected for each genotype and time point, and brains incubated with anti-PER, anti-TIM and anti-PDF antibodies. Immunofluorescence signals of whole-mounted brains were then quantified using the ImageJ software. **C.** PERIOD and TIMELESS oscillations in the s-LN_vs of *CG17350* mutant larvae. Mutant parent flies ($\Delta 17A$: *fon* single mutants, considered as controls, and $\Delta 22$: *CG17350/fon* double mutants) were leave to lay eggs for two days. Tubes were then entrained for 5 days in 12h:12h LD conditions, and after released to constant darkness at 25°C. Larvae were collected every 4 hours during the second day of constant darkness (DD2). For each genotype homozygous mutant larvae were isolated from heterozygous siblings by the absence of the *Act-GFP* marker that is associated to the balancer chromosome. Selected larvae were dissected and brains incubated with anti-PER, anti-TIM and anti-PDF antibodies. Immunofluorescence signals of whole-mounted brains were then quantified using the ImageJ software.

Fig. 2 Molecular effects of *CG17350*-RNAi expression

A. TIM is stabilized in *CG17350*-RNAi expressing flies during the subjective morning. Flies were entrained to standard LD cycles for at least 5 days and collected every 3 hours during the last day of LD and the subsequent first day of constant darkness. **B.** TIM is no more stabilized by *CG17350*-RNAi expression in a *per* null background. *per*⁰ and *per*⁺ flies were entrained to standard LD cycles for at least 5 days and collected at the first day of constant darkness. Non-oscillating hypo-phosphorylated TIM is present at all time points in a *per*⁰ background. *CG17350*-RNAi effects in a wild-type *per* background are also shown.

Genotype	Number ^a	% Rhythmicity ^b	Avg period (h) ± SEM	Power ^c
<i>w; ; UAS-17350-R2/+</i>	16	100	23,9 ± 0,05	234,3
<i>w; #18182</i>	16	100	23,8 ± 0,09	156,7
<i>yw; #23195</i>	15	93,3	23,9 ± 0,09	133,1
<i>w; ; gal1118/+</i>	16	100	24,0 ± 0,06	185,5
<i>w; ; gal1118/UAS-17350-R2</i>	16	100	27,0 ± 0,11	128,4
<i>w; ; gal1118/UAS-#30753</i>	16	100	24,3 ± 0,06	185,9
<i>w; UAS-#106106/+; gal1118/+</i>	16	100	23,8 ± 0,07	217,2
<i>w; UAS-dcr2/+; gal1118/+</i>	16	100	23,9 ± 0,05	192,5
<i>w; UAS-dcr2/+; gal1118/UAS-CG17350-R2</i>	16	100	23,9 ± 0,13	129,0
<i>w; UAS-dcr2/+; gal1118/UAS-#30753</i>	16	100	24,4 ± 0,07	189,5
<i>w; UAS-dcr2/ UAS-#106106; gal1118/+</i>	16	100	24,0 ± 0,07	195,1
<i>w; timgal4/+;</i>	15	100	24,0 ± 0,06	250,1
<i>w; timgal4/+; UAS-17350-R2/+</i>	27	100	25,9 ± 0,06	285,9
<i>w; timgal4/+; UAS-#30753</i>	16	100	24,1 ± 0,09	157,5
<i>w; timgal4/UAS-#106106</i>	15	100	23,9 ± 0,06	242,6
<i>w; timgal4/+; UAS-dcr2/+</i>	16	100	24,1 ± 0,08	157,4
<i>w; timgal4/+; UAS-dcr2/UAS-17350-R2</i>	16	100	27,0 ± 0,12	147,4
<i>w; timgal4/+; UAS-dcr2/UAS-#30753</i>	16	100	24,3 ± 0,06	203,4
<i>w; timgal4/UAS-#106106; UAS-dcr2/+</i>	16	100	24,3 ± 0,08	148,7

Table 1

Figure 1

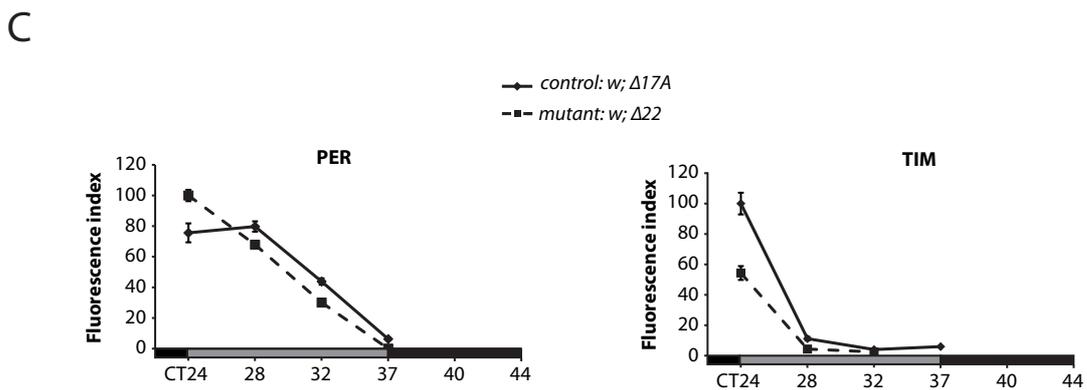
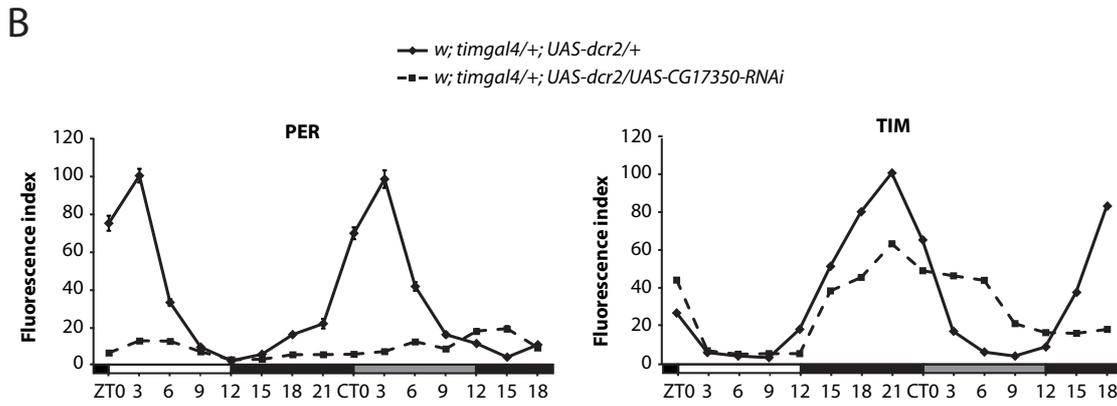
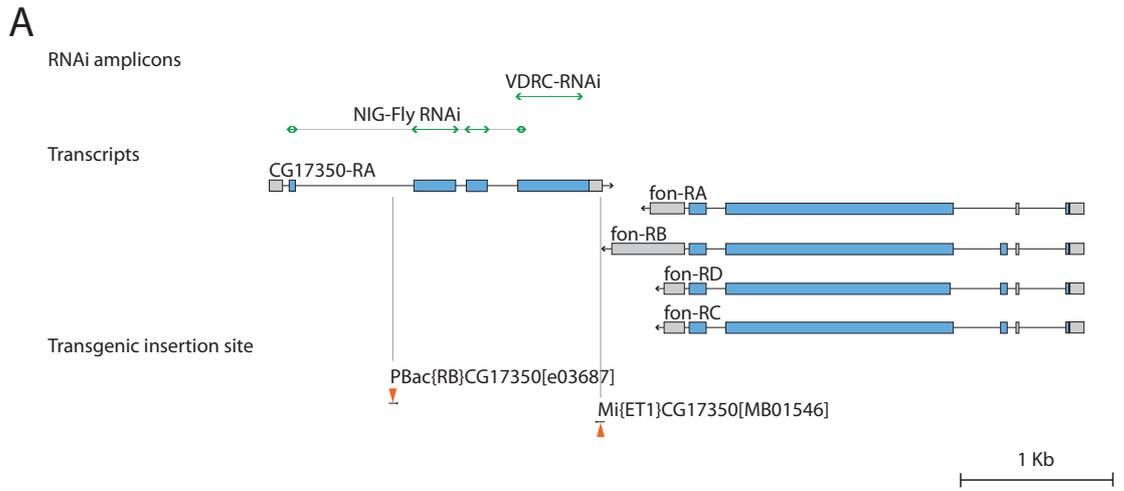
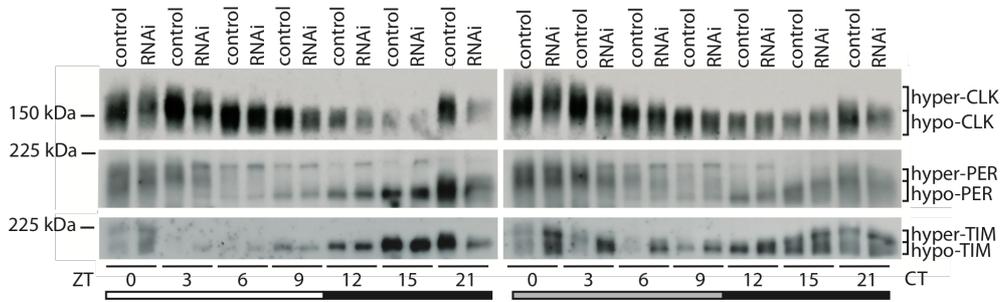
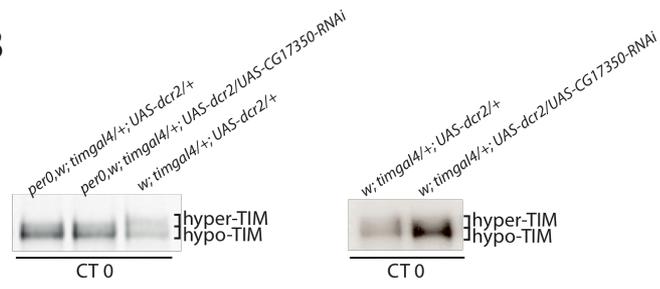


Figure 2

A



B



3. Discussion

Expression of the UAS-*CG17350*-RNAi using multiple clock-specific driver lines induced flies to adopt long-period rhythms that correlated with delayed PER and TIM protein oscillations in clock neurons. The molecular phenotype of *CG17350*-RNAi flies (strong stabilization of hypo-phosphorylated TIMELESS protein at the end of the cycle) supported a role for *CG17350* in promoting the TIM phosphorylation that triggers TIM proteasomal breakdown. Either a lowered kinase or, alternatively, an enhanced phosphatase activity can account for the hypo-phosphorylated TIM phenotype shown by RNAi expressing flies. The *CG17350* protein will then act as a kinase activator or a phosphatase inhibitor. *Per*⁰ flies showed constant levels of fast-migrating (hypo-phosphorylated) TIM, which, differently to a *per*⁺ background, were not stabilized after *CG17350*-RNAi expression. Hence, RNAi-mediated TIM stabilization depended on its partner PER. Alternatively, the *CG17350* protein works in the nucleus because, in the absence of PER, TIM cannot enter the nucleus and accumulate in the cytoplasm.

Although multiple RNAi transgenes targeting the *CG17350* gene were tested, only one showed a phenotype. The *CG17350* gene lies adjacent to the *fondue* (*fon*) gene, whose mutation causes pupal lethality (Bajzek et al., 2012). We did not succeed in generating proper *CG17350* null (excised) flies but preliminary analyses of double *CG17350*^{-/-}/*fon*^{-/-} mutants rather suggested that circadian oscillations in larval neurons were not affected after *CG17350* deletion. Protein oscillations in *fon* single mutant larvae were comparable to wild-type ones and *fon*-RNAi expression in clock cells had no effect on adult flies circadian locomotor rhythms. Therefore, *CG17350*-RNAi effects in adult flies were possibly not a direct consequence of *CG17350* downregulation, but rather affected expression of an “off-target” gene. *Rutabaga* (*rut*) is the first off-target gene predicted for *CG17350*-RNAi-mediated silencing. A *rut*-RNAi line (NIG-FLY #9533R2) as well as two *rut* mutants (*rut*^l, loss-of-function mutation and *rut*²⁰⁸⁰, P element insertion at the *rut* transcription starting region) were behaviorally tested and gave no altered phenotype (data not shown). The discrepancy between the absence of phenotype in *CG17350* null mutant larvae and the long-period effects of RNAi expression in adults can also be explained by the *CG17350* protein being irrelevant for the ticking of the larval clock and gaining circadian functions only at the adult stage.

The *CG17350* gene is essentially expressed at the adult stage, with enrichment in the head and testis, although at very low levels (Flybase). Yet, there is no prediction about the activity of the protein encoded by the *CG17350* gene. Moreover, this gene is peculiar to the

Drosophila family and has no counterpart in any other dipterans' genome (Flybase). Bioinformatic tools (ELM database) were consulted. The CG17350 polypeptide did not contain any predicted functional domain. Yet, an N-terminal signal peptide (SignalP/TargetP/PSORT2) was found, suggesting that CG17350 is possibly a secreted protein. However, the absence of any predicted transmembrane domain in the *CG17350* sequence rather suggests that CG17350 protein may localize inside certain organelles, like the endoplasmic reticulum, the Golgi, or endosomes.

Data shown here make an example of the limits of use of the RNAi technique for the silencing of genes. Indeed, the effects shown by expression of the *CG17350*-RNAi are more likely the consequence of the downregulation of a gene other than *CG17350*. Nevertheless, additional experiments could be done to corroborate or to disprove these preliminary results about a possible role for the CG17350 protein within the fly molecular clock. First, we need to verify a dampening of the *CG17350* mRNA quantities following *CG17350*-RNAi expression. If we confirm downregulation of *CG17350*, flies overexpressing a tagged version of CG17350 can be used for a co-immunoprecipitation assay that allows isolation of CG17350-interacting proteins. Analysis of the protein network associated with CG17350 will help to describe the biological and circadian role of the CG17350 protein.

IV. ARTICLE

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Coagulation and survival in *Drosophila melanogaster fondue* mutants

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ABSTRACT

Drosophila larval coagulation factors have been identified *in vitro*. Better understanding of insect hemolymph coagulation calls for experiments *in vivo*. We have characterized a *fondue* (*fon*) mutation and null alleles isolated by imprecise excision of a transposable element. Loss of *fon* was pupal lethal, but adults could be recovered by expressing the UAS:*fonGFP* construct of Lindgren et al. (2008). Despite their lethality, *fon* mutations did not affect larval survival after wounding either when tested alone or in combination with a mutation in the hemolectin clotting factor gene. This reinforces the idea of redundant homeostatic mechanisms in *Drosophila* larvae, and independent pleiotropic functions of the *fondue* protein in coagulation and a vital process in metamorphosis.

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1. Introduction

1.1. Coagulation in *Drosophila*

Coagulation is important to avoid excessive fluid loss and to block infection in insects (Theopold et al., 2002; Dushay, 2009; Cerenius and Soderhall, 2011; Loof et al., 2011). Better understanding of hemolymph clotting is thus important with respect to insect immunity and physiology. Despite its small size, the wealth of molecular genetics tools available in *Drosophila melanogaster* makes it a good system in which to study insect coagulation. Early experiments identified *Drosophila* larval clotting factors by direct isolation (Scherfer et al., 2004) and proteomics (Karlsson et al., 2004). Now it is important to study clotting *in vivo* to confirm and extend *in vitro* findings in living animals.

1.2. Coagulation study *in vivo*

The hemolectin (*hml*) gene was first identified as a gene expressed in hemocytes (Goto et al., 2001), and *hml* RNAi knockdown was shown to cause increased bleeding in larvae (Goto et al., 2003). Later studies confirmed *Hemolectin* as a major clotting protein, and RNAi knockdown of *hml* transcripts abolished clotting in *ex vivo* assays (Karlsson et al., 2004; Scherfer et al., 2004). Thus it was surprising that *hmlf03374* mutant larvae did not show any reduction in survival after wounding (Lesch et al., 2007). We designed an assay to test larvae for their ability to stop bleeding, and differences were found between the feeding and wandering

stages of third instar larvae that correlated with differences in the number of circulating hemocytes at the different stages (Chang et al., 2012). Using this assay, fewer *hmlf03374* larvae survived when wounded in the feeding stage of the third larval instar (Chang et al., 2012). Still, most wounded *hmlf03374* larvae survived. To explore why loss of *hml* had such a modest effect on wound survival, we set out to combine *hmlf03374* with mutations in another clotting factor gene, expecting it would further reduce coagulation and produce greater lethality.

1.3. *Fondue*

Fondue (*fon*) was one of the first clotting factors identified (Karlsson et al., 2004; Scherfer et al., 2004). RNAi knockdown of *fon* showed effects in *ex vivo* larval clotting assays and was lethal at the pupal stage, causing pupae to be distinctively elongated and curved (Scherfer et al., 2006; Lindgren et al., 2008). A *fon* cDNA-*GFP* fusion construct was shown to fluorescently label the clot and produce a distinctive striped pattern in larvae (Lindgren et al., 2008). However *fon* mutants were not available at that time, so the construct could not be tested for production of functional *fondue* protein.

We have now characterized MB11923 flies with a *Minos* transposable element insertion in the first intron of the *fon* gene. We confirmed that this insertion resulted in a *fon* mutation and confirmed that *fon* is a vital gene. We isolated *fon* null alleles by imprecise excision of the MB01546 *Minos* transposon inserted in *CG17350*, adjacent to the *fon* gene. We now show that the UAS:*fonGFP* fusion construct (Lindgren et al., 2008) produces functional *fondue* protein that can partially rescue adult viability. Similar to *fon* RNAi knockdown (Scherfer et al., 2006; Lindgren

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et al., 2008), strong hypomorphic and null *fon* alleles caused lengthening of the strands that could be drawn out of coagulating hemolymph. However, complete loss of *fon* did not cause any reduction in larval wound survival alone or in combination with *hml*^{f03374}. Our results provide additional evidence for redundant homeostatic mechanisms in *Drosophila* larvae, and highlight the need for additional *in vivo* assays to shed light on insect hemolymph coagulation.

2. Methods

2.1. *Drosophila* strains

Flies bearing the MB11923 *Minos* element insertion in the first intron of *fon* (Fig. 4a) and other stocks were obtained from the Bloomington *Drosophila* Stock Center. A stock with *Minos* element MB01546 insertion in CG17350 adjacent to *fon* was obtained from the *Drosophila* Genetic Resource Center, Kyoto Institute of Technology. The *Df(2L)Exel6043* deficiency uncovers *fon* (Flybase). Flies were maintained on mashed potato, sugar, yeast medium. Outcrossing of MB11923 was performed by mating *w*¹¹¹⁸; MB11923/+ females to *w*¹¹¹⁸ males iteratively for 10 generations. Flies bearing the MB11923 insertion were recognized by GFP fluorescence in their eyes from the recombinant *Minos* element (Bellen et al., 2004). Homozygous *fon*^Δ and *fon*^Δ/*Df* larvae, including those bearing *daGAL4>>UAS:fonGFP*, were distinguished from larvae bearing the *CyOActGFP* balancer by its pattern of fluorescence (Fig. 6).

2.2. *fon* Excisions

Deletions of the *fon* locus were generated by excision of the MB01546 *Minos* transposable element. The *Mi(ET1)CG17350*^{MB01546} allele is a *Minos* insertion in the non-coding 3' end of the CG17350 gene, not far from the C-terminus of the adjacent *fon* gene (Fig. 4a). Flies homozygous for *Mi(ET1)CG17350*^{MB01546} are viable and fertile and did not show any gross morphological defects. The *Minos* element was remobilized by crossing *Mi(ET1)CG17350*^{MB01546} flies with P{hsILMiT} flies that provide a *Minos* transposase source driven by a heat-shock promoter. To improve deletion rate, we used a genetic background carrying a mutation of the *mus309* gene, which increases both the number and size of the flanking genomic DNA deletions induced by the excision of *Minos* elements (WitSELL et al., 2009). The *mus309* gene encodes a DNA helicase involved in homology-directed repairs of double-strand breaks that typically occur after transposon excision. Crosses were set as to obtain a *mus309*^{D2}/*mus309*^{fl} heteroallelic mutant background together with the *Minos* element insertion and the transposase. Embryos were heat-shocked daily for 1 h at 37 °C until hatching. Eighty stable independent lines were established that had lost the EGFP marker of the *Mi(ET1)* insertion. Of these, 14 jump-outs were homozygous lethal; three at the embryonic stage and eleven at pupal stage. The genomic DNA of larvae from the pupal lethal lines was analyzed by PCR with primers flanking the insertion site (Fig. 4a). Seven lines were deleted for both CG17350 and *fon*, and four lines were deleted for *fon* only without affecting the CG17350 exons. PCR products of CG17350, *fon* and CCG17549 for 3 independent excision lines are shown in Fig. 4b. Δ17A and Δ24A larvae show deletion of *fon* only. The Δ27A line is deleted for both CG17350 and *fon*. *w*¹¹¹⁸ Larvae were used as positive controls. PCR primers were: CG17350-496: 5'-CCTGTTGATAITGCTCTCGG-3', CG17350-497: 5'-TCTCTTCTGGCTTGCTATCCC-3', *fon*-541: 5'-GAT-ATCAACACITTCGCTCTCGC-3', *fon*-542: 5'-TGATGACTCTGCTCTGG-GAAAAC-3', *fon*-543: 5'-GGGAGCTGGCAGAGITCAAAG-3', CG 17549-539: 5'-TCTAGAGTGCACCGAATACAG-3', CG17549-540: 5'-TGTGTTGATGAGTCTCTGAATG-3'.

2.3. Larval staging

Feeding stage larvae were isolated using blue food as described in (Chang et al., 2012). Briefly, larvae were kept on medium with 0.5% bromophenol blue, and third instar feeding stage larvae were identified by their visibly blue guts. In contrast, third instar larvae that had entered the wandering stage had stopped eating and begun clearing their guts that consequently were paler shades of blue.

2.4. Spaghetti assay

The spaghetti assay was performed as described by Bidla et al. (2005). Briefly, wandering stage larvae were washed in deionized water, and hemolymph was collected on microscope slides by tearing open larvae with fine forceps. Hemolymph from 5 larvae was pooled, and strands were pulled up like spaghetti from a pot as the hemolymph began to clot. In place of a really small spaghetti fork, we used acupuncture needles. Strand length before breaking varied to some degree between different investigators (as demonstrated by different lengths for *w*¹¹¹⁸ controls between different experiments shown in Figs. 3 and 5), but was consistent for each person within each experiment, as illustrated by the low SEM values for each individual experiment.

2.5. Wounding assay

The wounding assay was described in (Chang et al., 2012). Briefly, feeding stage larvae were isolated from blue food vials, washed in deionized water, and gently dried with tissue. Larvae in groups of 20 were wounded in a caudo-lateral region with a fine tungsten needle. Wounded larvae were additionally stressed by leaving them on dry tissue for 45 min at room temperature. The tissue and larvae were then laid over fly medium in a petri dish and incubated at 25 °C for 24 h, after which survival was scored. Each measurement thus represents the mean survival of 20 larvae.

3. Results

3.1. *fon*^{MB11923}

MB11923 was described as a lethal mutation caused by the insertion of a *Minos* transposable element in the first intron of the *fon* gene (Flybase) (Fig. 4a). As this insertion does not affect the coding sequence of the *fon* gene and lethality could result from other chromosomal aberrations created during transposable ele-



Fig. 1. *fon*^{MB11923} Pupal phenotype. A wild type pupa is on the left. The *fon*^{MB11923} pupa at center shows elongation, and the *fon*^{MB11923} pupa on the right shows elongation and bending.

ment mobilization (O'Keefe et al., 2007), we first sought to demonstrate that the MB11923 insertion caused a bona fide allele of *fon*. We observed that the homozygous MB11923 pupae were elongated and showed the characteristic bend described for *fon* RNAi knockdown lines (Scherfer et al., 2006; Lindgren et al., 2008) (Fig. 1). Similarly, MB11923/*Df* larvae did not show marked lethality until pupariation, after which no adults emerged (data not shown). To confirm that the cause of lethality mapped to the insertion, MB11923 was outcrossed for ten generations (Section 2.1). Outcrossed MB11923 was still homozygous lethal at the pupal stage. This demonstrated that MB11923 was an allele of *fon* (*fon*^{MB11923}) and that *fon* is a vital gene.

Having shown that *fon*^{MB11923} mutants exhibited phenotypes similar to those of *fon* RNAi knock-downs, we tested *fon*^{MB11923} for effects on survival of wounding at the feeding stage. Unlike *hml*⁰³³⁷⁴ however, the survival of *fon*^{MB11923} was not significantly different from wild type larvae (Fig. 2).

To further assess the coagulation phenotype of *fon*^{MB11923} larvae, we performed spaghetti assays (Section 2.4). Like the effects of *fon* RNAi knockdown (Scherfer et al., 2006; Lindgren et al., 2008), hemolymph from *fon*^{MB11923} larvae produced longer strands than wild type controls (Fig. 3). We also compared spaghetti assay results from wild type larvae in the feeding and wandering stages of the third instar. Significantly longer strands could be drawn from the hemolymph of wild type feeding stage larvae than from the hemolymph of wandering stage larvae. The latter have more hemocytes in circulation (Chang et al., 2012), and hence are able to form a denser, more brittle clot, leading to strands breaking earlier, at shorter lengths in the spaghetti assay.

3.2. *fon* null alleles

The difference in strand length between *fon*^{MB11923} homozygotes and *fon*^{MB11923}/*Df* larvae (Fig. 3) suggested that some *fon* protein function might still be present in *fon*^{MB11923} larvae. This led us to screen for *fon* null alleles by mobilizing the *Minos* element in *fon*^{MB11923}. We crossed *fon*^{MB11923} to P(hslLMiT) flies bearing a

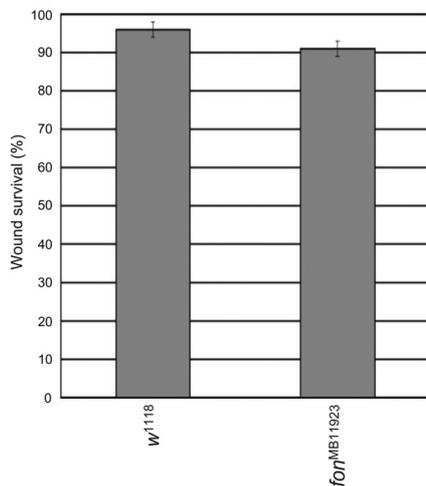


Fig. 2. Wound survival of *fon*^{MB11923} larvae (Section 2.5). The mean percent survival of larvae wounded in the feeding stage are shown, with error bars representing SEM. Each value represents the mean of five trials of 20 animals each (100 larvae, n = 5). While *fon*^{MB11923} mutant larvae survived wounding less than wild type controls, this difference was not statistically significant ($P > 0.05$ by Student's *t* test).

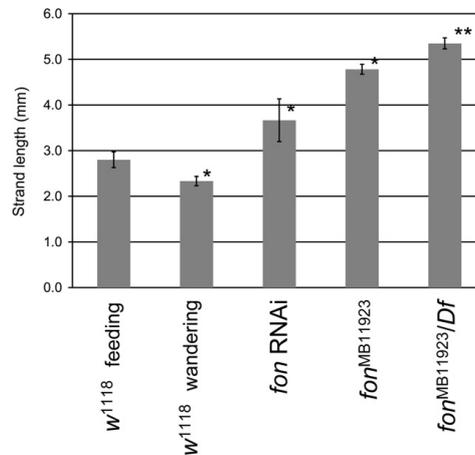


Fig. 3. Spaghetti assay results for *fon*^{MB11923}. RNAi knockdown of *fon* led to strand lengthening by this assay (Scherfer et al., 2006; Lindgren et al., 2008). Longer strands could be drawn from *fon*^{MB11923} larval hemolymph, while still longer strands could be drawn from *fon*^{MB11923}/*Df* hemolymph. This suggested that while *fon*^{MB11923} had a greater effect than RNAi knockdown, the larvae still retain some residual *fon* coagulation function. In addition, we compared wildtype larvae in the feeding and wandering stages and demonstrated that like wound survival (Chang et al., 2012), hemolymph from different stage larvae showed different strand lengths. Significant differences were found between each group ($P < 0.05$ by *t* test) indicated by asterisks, while the difference between *fon*^{MB11923} and *fon*^{MB11923}/*Df* was highly significant ($P < 0.01$) as indicated by double asterisks. Error bars represent SEM, and n = 6 for each group.

Minos transposase source driven by a heat-shock promoter following the protocol of Metaxakis et al. (2005). Several fully viable excision lines were recovered (data not shown). This was consistent with outcrossing results (Section 3.1), demonstrating that the lethality of *fon*^{MB11923} homozygotes was specific to loss of *fon* and not caused by other genetic defects in this strain.

Imprecise excisions were generated from another *Minos* element MB01546, located in *CG17350* and close to *fon* (Section 2.2, Fig. 4a). Excisions that were homozygous lethal at the pupal stage were tested for deletion of *CG17350* and *fon* exons by PCR. As shown in Fig. 4b, $\Delta 17A$ and $\Delta 24A$ strains showed deletion of *fon* only, while the $\Delta 27A$ strain was deleted for both *CG17350* and *fon*.

Pupae of the *fon* excision stocks showed the characteristic lengthening and bending phenotype (data not shown). Spaghetti assays showed that long strands could be drawn out of the hemolymph of all three *fon* excision lines (Fig. 5). As expected for amorphic alleles, no difference in strand length was observed between *fon* Δ^{24A} homozygotes and *fon* Δ^{24A} /*Df* larvae.

3.3. *fon* Rescue: *fon* Δ^{17A} and *fon* Δ^{27A}

To confirm that the lethality of the *fon* excision lines was specifically caused by loss of *fon* and not the disruption of other genes, and to test if the *UAS::fonGFP* construct (Lindgren et al., 2008) produced functional *fon* protein, we generated *fon* Δ^{17A} and *fon* Δ^{27A} strains bearing the *daGAL4* driver and the *UAS::fonGFP* responder constructs (Fig. 6). As shown in Table 1, adult flies emerged in these vials. This showed that *daGAL4*-driven ubiquitous expression of the *UAS::fonGFP* construct could rescue the lethal *fon* null excision alleles, even if only partially. Note however that *daGAL4* \gg *UAS::fonGFP* expression did not rescue the long-strand phenotype of *fon* Δ^{17A} and *fon* Δ^{27A} larvae in spaghetti assays (data not shown). This was probably due to the timing and amount of

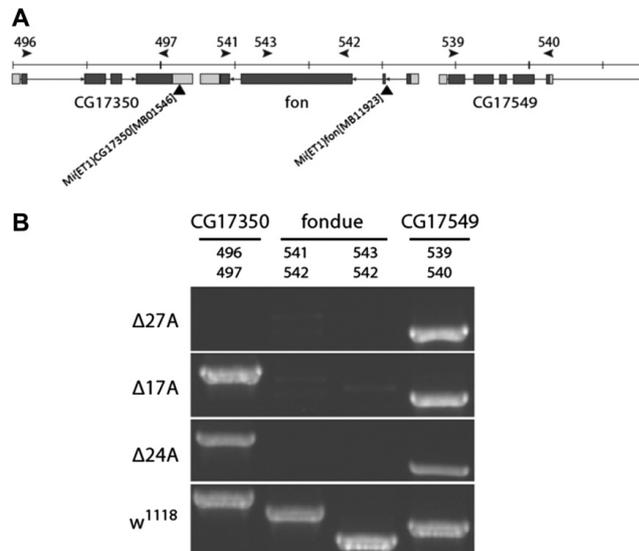


Fig. 4. MB11923, MB01546, and *fon* null excisions. (A) Schematic drawing adapted from flybase showing the region surrounding *fon*, the locations of *Minos* elements MB11923 in the first intron of *fon*; MB01546 in the last exon of *CG17350*; and *CG17549*. Exons are represented as boxes, with dark grey filled boxes indicating coding regions, and pale grey filled boxes indicating non-coding regions. The approximate locations of PCR primers used to characterize excision stocks are indicated as arrowheads. (B) Photograph of electrophoresis gels showing PCR products from three *fon* null excisions and w¹¹¹⁸ wild type as described in Section 2.2. Genes tested and the primer pairs used are indicated above each lane.

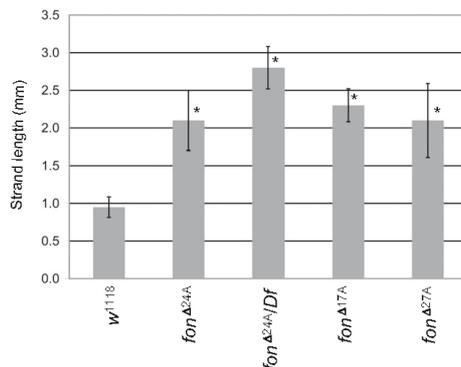


Fig. 5. Spaghetti assay results for *fon* null alleles. Each value represents the mean of five trials of 20 animals each (100 larvae, $n = 5$), and error bars represent SEM. As expected for a *fon* amorph, longer strands could be drawn from coagulating hemolymph from *fon* null mutants than control larvae ($P < 0.01$ by Student's *t* test), marked by asterisks. Although it appears that still longer strands could be drawn from hemolymph from *fon*^{Δ24A}/*Df* larvae, this difference was not statistically significant. Note that the difference in scale between these data and those shown in Fig. 2 are explained by these experiments being performed by different authors (see also Section 2.4).

UAS:fonGFP fusion protein expression in these larvae, and this was not investigated further.

3.4. *fon*; *hml* Double mutants

Having generated and characterized *fon* null alleles, we were now in position to test our original question, whether loss

of *hemolectin* and *fondue* together led to marked reduction in the survival of wounded feeding stage larvae. In the event, loss of *fon* had no measurable effect on survival alone or in combination with *hml*^{f03374} as shown in Fig. 7.

4. Discussion

We have demonstrated that *Minos* insertion MB11923 caused a mutation of *fon*. Surprisingly, while *fon*^{MB11923} is fully lethal, its effect on coagulation as measured by spaghetti assay suggested some residual *fondue* protein activity is still present in *fon*^{MB11923} homozygous larvae. The *fon*^{MB11923} allele is thus a strong hypomorph. Null *fon* alleles were isolated by mobilizing the MB01546 *Minos* element inserted in *CG17350*, adjacent to *fon* (Fig. 4a). These were confirmed as excisions of *fon* by PCR (Fig. 4b), and viability was partially rescued by driving ubiquitous expression of *UAS:fonGFP* (Lindgren et al., 2008) with *da-GAL4* (Fig. 6 and Table 1).

We tested *fon* alleles for effects on coagulation by spaghetti assay and by wound survival. As expected, these mutants displayed stronger phenotypes than previously found in *fon* RNAi knock-down experiments (Scherfer et al., 2006; Lindgren et al., 2008). None of the *fon* null mutations interfered with ability to draw-out strands from coagulating hemolymph, but only led to longer strands. This is consistent with a role for the *fondue* protein in clot cross-linking (Lindgren et al., 2008), and argues that *Fondue* is not required for coagulation per se.

We were unable to show that loss of *fon* alone or in combination with *hml*^{f03374} reduced the survival of wounded larvae, even though our protocol was designed to detect defects in coagulation *in vivo* (Chang et al., 2012). In a sense our results validate the importance of studying coagulation in whole living animals and provide further evidence that redundant hemostatic mechanisms are present in *Drosophila* larvae (Lesch et al., 2007;

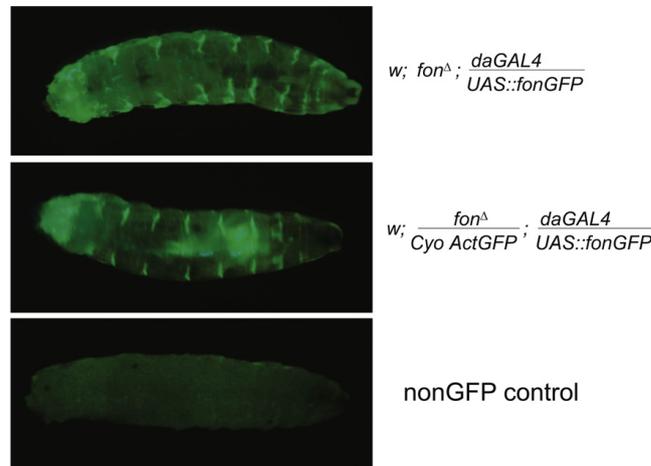


Fig. 6. Photomicrographs of *daGAL4>>UAS::fonGFP* larvae. The *w; fon Δ ; daGAL4/UAS::fonGFP* larvae showed the characteristic striped pattern described by Lindgren et al. (2008). The heterozygous *w; fon Δ ; Cyo ActGFP; daGAL4/UAS::fonGFP* larvae showed bright imaginal disc staining caused by the *CyoActGFP* balancer. This enabled us to separate balanced heterozygotes from *fon Δ* homozygous larvae. An image of a nonGFP larva is included.

Table 1

Rescue of *fon Δ* lethality by *daGAL4>>UAS::fonGFP*. 30 homozygous *fon Δ ^{17A}* or *fon Δ ^{27A}* larvae bearing both *daGAL4* and *UAS::fonGFP* were set in vials at 25 °C for three trials of each genotype. While no adults emerged from the vials set with homozygous *fon* larvae not expressing *UAS::fonGFP*, vials set with *fon* larvae that also carried *daGAL4/UAS::fonGFP* produced adults in the numbers shown. Thus the *UAS::fonGFP* construct (Lindgren et al., 2008) produced functional *fondue* protein, and its expression driven by *daGAL4* at 25 °C was enough to partially rescue *fon* lethality.

Genotype	Adult survivors (mean + SD)
<i>w¹¹¹⁸</i>	29 + 1
<i>w; Δ17A</i>	0
<i>w; Δ17A; daGAL4/UASfonGFP</i>	7 + 4
<i>w; Δ27A</i>	0
<i>w; Δ27A; daGAL4/UASfonGFP</i>	5 + 1

Chang et al., 2012). These probably include the plugs of debris and hemocytes observed following wounding (Galko and Krasnow, 2004). Investigating the mechanisms of plug formation, its relationship to coagulation, and its role in hemostasis are topics for further research.

How loss of *fondue* causes pupal lethality remains an interesting problem. It is unlikely to involve coagulation, particularly since *hml⁰³³⁷⁴* mutants (with stronger clotting effects than *fon* mutants) are fully viable. *Fondue* is a major substrate of transglutaminase in the clot (Lindgren et al., 2008). Transglutaminase is involved in cuticle maturation in addition to coagulation, and RNAi knockdown of transglutaminase was also pupal lethal, but *fondue* protein was not detected in the pupal cuticle (Shibata et al., 2010). Alternatively, the fluorescent stripes seen in *daGAL4>>UAS::fonGFP* larvae (Fig. 6) resemble the embryonic stripes of *tiggrin* expression (Fogerty et al., 1994). *tiggrin* was also identified as a clotting factor (Karlsson et al., 2004; Scherfer et al., 2004). Notably, like *fon* mutants, *tiggrin* mutants are also pupal lethal with pupal lengthening, and this was shown to be caused by the loss of *tiggrin* protein in myotendinous junctions (Bunch et al., 1998). Thus *fondue* might be involved in myotendinous junctions as well as clotting like *tiggrin*. Experiments exploring this are underway.

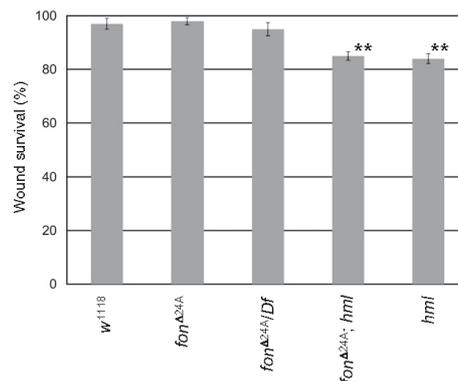


Fig. 7. Wound survival of *fon* null mutants alone and in combination with *hml*. The mean percent survival of larvae wounded in the feeding stage are shown. Each value represents the mean of five trials of 20 animals (total 100 larvae, *n* = 5), and error bars represent SEM. The loss of *fon* in all three excision lines did not cause any reduction in survival following wounding, despite the clear effect of these deletions on coagulation demonstrated by spaghetti assay (Fig. 4). Although *hml* mutant larvae showed significantly less survival (*p* < 0.01) as shown before (Chang et al., 2012), combining this *hml* allele with *fon Δ ^{24A}* did not produce an additional effect on wound survival. Both *fon Δ ^{24A}; hml⁰³³⁷⁴* and *fon Δ ^{24A}; hml⁰³³⁷⁴* double mutants are thus marked with double asterisks to indicate the difference from *w¹¹¹⁸* control larvae.

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Résumé

Les mécanismes moléculaires contrôlant les rythmes circadiens sont conservés parmi les organismes des différents règnes (plantes, animaux et champignons). Ils se composent de boucles de rétroaction où un complexe d'activation transcriptionnelle, l'hétérodimère CLOCK/CYCLE (CLK/CYC) chez la drosophile, entraîne l'expression des gènes codant pour les répresseurs de son activité, *period (per)* et *timeless (tim)* chez la drosophile. La période de l'oscillateur dépend en grande partie de mécanismes post-transcriptionnels qui régulent l'accumulation et l'activité des composantes positifs et négatifs de la boucle. Un certain nombre de composants qui modifient la stabilité et l'activité des protéines PER, TIM, CLK et CYC ont été isolés mais de nombreux aspects du fonctionnement de l'oscillateur reste encore mal compris.

Dans le cadre de la recherche de nouveaux composants de l'horloge circadienne, nous avons réalisé un crible comportemental basé sur l'expression ciblée de transgènes ARNi dirigés contre environ 6000 gènes soit la moitié du génome de *Drosophila melanogaster*. Cinquante-quatre nouveaux gènes putativement impliqués dans l'horloge ont pu être identifiés. Au cours de ce travail, j'ai étudié le rôle de deux d'entre eux, sélectionnés pour les forts défauts comportementaux que provoque la baisse de leur expression par ARNi.

Le gène *CG12082* de la drosophile est l'orthologue de l'Ubiquitin-Specific Protease 5 (USP5) chez l'homme. La dérégulation d'*Usp5* retarde les oscillations de la protéine PER dans les neurones d'horloge et allonge la période du rythme d'activité locomotrice des mouches. Chez les mouches ARNi *Usp5*, des formes à haut poids moléculaire des protéines PER et TIM s'accumulent le matin, alors qu'elles sont normalement dégradées chez les contrôles. On a pu montrer que USP5 participe directement à la dégradation de la protéine PER, indépendamment de TIM. En accord avec le rôle décrit pour l'orthologue humain, USP5 serait susceptible de contrôler la dégradation des protéines par son activité de dépolymérisation des chaînes libres d'ubiquitine présents dans la cellule, qui peuvent entrer en compétition avec les protéines ubiquitinylées pour leur reconnaissance par le protéasome et bloquer leur dégradation.

La plus grande partie de ma thèse a porté sur un second gène, *Strip*, dont les fonctions sont encore inconnues. La protéine STRIP est trouvée dans des complexes de la phosphatase PP2A, complexes particuliers qui contiennent une nouvelle sous-unité régulatrice de PP2A, CKA. La dérégulation des gènes *Strip* et/ou *Cka* induit un allongement de la période du rythme comportemental. D'un point de vue moléculaire, elle induit l'accumulation matinale de formes hyper-phosphorylées de la protéine CLK. Les formes hyper-phosphorylées de CLK sont transcriptionnellement inactives et la transcription des gènes *tim* et *vriille*, cibles de CLK, est fortement réduite dans les mouches ARNi *Cka*. La dérégulation de la sous-unité catalytique de PP2A produit également une hyper-phosphorylation de CLK le matin, indiquant que l'action des complexes PP2A contenant CKA rend compte de la majorité de l'action des complexes PP2A totaux. Par ailleurs, il a été montré que des complexes PP2A contenant une autre sous-unité régulatrice, WDB, déstabilisent CLK dans des cellules en culture. Nous avons observé que la dérégulation de WDB affecte la stabilité du CLK également dans la mouche adulte, sans toutefois induire un défaut de phosphorylation détectable. En conclusion, deux complexes PP2A différents agissent sur la protéine CLK: les complexes PP2A/CKA/STRIP contrôlent la déphosphorylation de CLK et sa réactivation, tandis que les complexes PP2A/WDB affectent la stabilité de CLK indépendamment. Ces résultats enrichissent l'étude de la régulation post-traductionnelle de la protéine CLK, qui est à ce jour largement inconnue.

Summary

The molecular mechanisms underlying circadian rhythms are conserved among organisms and consist of feedback loops where a transcriptional activating complex, the CLOCK (CLK)/CYCLE (CYC) heterodimer in *Drosophila*, drives the expression of the genes encoding the repressors of its activity, the *period* (*per*) and *timeless* (*tim*) genes in *Drosophila*. The pace of the oscillator largely depends on post-transcriptional mechanisms that regulate the accumulation and activity of both the positive and negative components of the loop. A number of interacting partners that modify core clock components have already been isolated but more are expected.

To isolate new clock components, we performed a behavioral screen based on the targeted expression of RNAi transgenes directed to about 6000 genes (half of the *Drosophila* genome). Fifty-four putative new clock genes were identified, including some already reported to play a role in the *Drosophila* clock, thus validating the screen. In this work, I investigated the role of two new genes, selected for the strong behavioral defects induced by the expression of the corresponding RNAi.

The *CG12082* gene encodes the *Drosophila* ortholog of the human Ubiquitin-Specific Protease 5 (USP5). Downregulation of *usp5* in clock cells lengthens the period of both locomotor activity rhythms and PER protein oscillations in the clock neurons. High molecular weight forms of PER and TIM proteins accumulate during the morning after *usp5* knockdown, whereas these forms are degraded in controls. In addition, TIM is not stabilized in the absence of PER, whereas PER still accumulates in the absence of TIM. Therefore, USP5 directly participates to the degradation of the PER protein and, secondarily, of the TIM protein, at the end of the cycle. According to the role described for its human ortholog, USP5 likely controls protein degradation through the disassembling of the unanchored polyubiquitin chains that could compete with ubiquitinated proteins for proteasome recognition and subsequent breakdown.

The majority of the work was dedicated to an unknown gene that we named *Strip*, in reference to its human homolog. We show that the STRIP protein interacts with Connector of Kinase to AP-1 (CKA), a novel regulatory subunit for the PP2A phosphatase. Downregulation of both *Strip* and/or *Cka* causes long-period behavioral rhythms and induces the accumulation of high molecular weight forms of CLK in the morning. Downregulation of the gene encoding the catalytic subunit of PP2A also produces hyper-phosphorylated CLK in the morning, indicating that CKA/STRIP PP2A complexes dominate over other PP2A complexes for CLK regulation. Hyper-phosphorylated CLK forms are transcriptionally inactive. Accordingly, transcription of the *tim* and *vriille* CLK targets is strongly reduced in *Cka*-RNAi flies. PP2A complexes containing the WDB regulatory subunit were already shown to affect CLK stability in culture cells. We show that *wdb* downregulation also affects the stability of CLK in fly head extracts, but has no apparent effects on CLK phosphorylation. Therefore, we could describe two different PP2A complexes acting on the CLK protein: PP2A/CKA/STRIP complex controls CLK dephosphorylation and reactivation, whereas PP2A/WDB affects CLK stability, independently.