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TESI DI LAUREA

# Search for new genes involved in Drosophila circadian rhythmicity: behavioural analysis of putative CRYPTOCHROME interacting proteins and molecular characterization of the ubiquitin protease USP5

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# ABSTRACT

Circadian (~24 hours) clocks enable organisms to anticipate daily environmental changes with suitable physiological and behavioural responses. By definition, they keep functioning in the absence of external stimuli but use environmental cues to synchronize to day-night cycles. Light-synchronization of *Drosophila* clocks mostly goes through the CRYPTOCHROME (CRY) photoreceptor. In order to elucidate the molecular function of CRY, a number of putative CRY interacting proteins were isolated. The expression of these genes was inhibited by RNAi to search for a *cry* mutant clock phenotype, hence behavioural rhythmicity in constant light conditions. Circadian oscillators are based on transcriptional feedback loops and modulated by post-translational protein modifications, which set the pace of the clock. Ubiquitin Specific Peptidase 5 (USP5) is a new enzyme involved in the *Drosophila* clock. In constant darkness conditions, *usp5*-RNAi expression in the brain clock neurons induces a lengthening of the clock proteins oscillations, which slows-down behavioural rhythms. Clock proteins accumulate abnormally, suggesting an alteration of the degradation program. Since Usp5 effects are very reduced in light:dark conditions, our data suggest its specific involvement in the circadianly regulated degradation of the clock proteins.

# RIASSUNTO

Una delle caratteristiche fondamentali per l'evoluzione di un organismo è la capacità d'adattamento alle variazioni dell'ambiente in cui vive. Il moto di rotazione terrestre ha indotto la gran parte degli organismi a evolvere un sistema capace di anticipare le variazioni giornaliere di luce e temperatura per adattarne i propri ritmi comportamentali e fisiologici, al fine di aumentare la fitness. Nell'uomo, l'orologio circadiano controlla l'alternarsi del ritmo sonno-veglia, il livello d'attenzione e le performance intellettuali, così come la secrezione d'ormoni ed il metabolismo. Alterazioni del ritmo circadiano causate, ad esempio, da turni notturni di lavoro oppure jet-lag, provocano all'organismo un rilevante stress fisiologico e possono condurre a disordini come insonnia, depressione, obesità e predisporre al cancro. *Drosophila melanogaster*, il moscerino della frutta, si è rivelato un ottimo modello per identificare le basi molecolari dei ritmi circadiani. In particolare, il moscerino condivide con i mammiferi omologhi per la gran parte dei geni coinvolti nell'organizzazione molecolare del clock. Decifrare gli elementi che regolano il battito dell'orologio in *Drosophila* è quindi un importante contributo per la comprensione dei meccanismi che controllano i ritmi circadiani nell'uomo. *Drosophila*, come altri organismi, possiede orologi multipli. L'orologio situato nel cervello controlla i comportamenti complessi, come, nel moscerino, la locomozione. Gli orologi periferici soddisfano la regolazione circadiana di un organo o un tessuto. Da un punto di vista molecolare, in ciascun orologio, la ciclicità è garantita dall'azione di un meccanismo a feedback, secondo il quale una proteina inibisce la propria espressione. In *Drosophila*, i fattori di trascrizione CLOCK (CLK) e CYCLE (CYC) attivano l'espressione dei geni *period* (*per*) e *timeless* (*tim*) alla fine del giorno. Durante la notte, le proteine PER e TIM si accumulano nel citoplasma, si associano ed entrano nel nucleo, dove inibiscono l'attività trascrizionale di CLK/CYC. Al mattino, PER e TIM sono infine degradati, permettendo l'inizio di un nuovo ciclo.

Per definizione, gli orologi circadiani sostengono il ritmo anche in assenza di condizionamenti ambientali, ma si servono di segnali esterni, come luce e temperatura, per sincronizzarsi. In *Drosophila*, nella sincronizzazione ai cicli luce-buio gioca un ruolo cardine il fotorecettore alla luce blu, CRYPTOCHROME (CRY). Questa molecola è direttamente espressa nella gran parte dei neuroni orologio del cervello, e in molte altre cellule del corpo del moscerino, permettendo loro di regolarsi autonomamente con i cicli luce:buio esterni. Una volta foto-attivato, CRY riconosce la proteina TIM ne induce la degradazione tramite proteasoma. Quest'azione permette di resettare l'orologio molecolare in conformità alla durata del giorno. Sebbene sia ampiamente condiviso il meccanismo generale, i dettagli molecolari che regolano la stabilità della proteina CRY e il suo riconoscimento di TIM non sono ancora ben chiari. Con questo proposito, tramite analisi con il doppio ibrido di lievito e co-immunoprecipitazione, è stata stilata una lista di putativi interattori di CRY. L'espressione di ciascuno di questi geni è stata inibita in tutte le cellule che esprimono *tim* mediante RNA interference (RNAi) attraverso il sistema UAS/GAL4. I moscerini transgenici sono stati quindi testati per un tipico fenotipo mutante di *cry*, la ritmicità locomotoria in condizione di luce costante. Sfortunatamente, nessuno dei geni isolati sembra coinvolto nella foto-periodicità circadiana, ma ulteriori test sono necessari per escluderne l'implicazione. Una possibilità è il coinvolgimento della proteina-target negli orologi periferici, che non controllano l'attività locomotoria: in questo caso l'effetto dell'interference non è apprezzabile tramite i test comportamentali classici (locomozione). L'optomotor test permette di vagliare l'integrità del sistema visivo del moscerino: un sottogruppo selezionato di putativi partner di CRY verrà testato per un possibile coinvolgimento di CRY (e dei suoi partner) nella percezione del movimento.

A livello molecolare, specifiche modificazioni post-traduzionali controllano la stabilità, la localizzazione subcellulare e l'attività delle proteine dell'orologio. Tali variazioni sono cruciali per la determinazione dell'ampiezza, fase e periodo del ciclo. Una buona parte degli studi attuali sul clock è focalizzata sull'identificazione delle proteine che regolano queste modificazioni e sulla loro funzione. Il problema maggiore nell'identificazione di nuovi geni partner

dell'orologio risiede nel fatto che la gran parte di essi codifica per proteine che svolgono molteplici ruoli all'interno della cellula, e possono essere necessarie durante lo sviluppo dell'individuo. Il sistema UAS/GAL4 è un'ottima tecnica per modificare l'espressione di geni la cui forte mutazione è letale, e può essere usato per indurre l'espressione di costrutti RNAi in specifiche cellule target. In collaborazione con la banca di linee RNAi giapponese NIG-FLY, l'equipe di F. Rouyer ha avviato un progetto di screening su larga scala del genoma di *Drosophila* alla ricerca di nuovi geni dell'orologio. L'RNAi contro l'mRNA del gene endogeno è stato specificatamente indotto nei neuroni dell'orologio cerebrale che controllano la ritmicità locomotoria in condizioni di buio costante, gli s-$LN_v$s (small lateral ventral neurons). Tra i geni risultati positivi (vale a dire, la cui down-regolazione per RNAi induce una variazione nella lunghezza del periodo del ciclo, o arritmicità), mi sono occupata dell'analisi di USP5, un'ubiquitina proteasi. In condizioni di buio costante, la ridotta espressione di questo gene causa nei moscerini un periodo di attività locomotoria di circa 28-30 ore. L'analisi delle quantità di proteine PER e TIM negli s-$LN_v$s ha confermato che esse oscillano con un periodo allungato di circa 4 ore rispetto al normale (controlli), e con una ridotta ampiezza. Lo studio mediante western blot su estratti di teste ha rivelato un'anomala persistenza di PER, TIM e CLK alla fine del giorno – inizio della notte, indice di una mancata degradazione. Inoltre, si assiste alla stabilizzazione della forma ipo-fosforilata e trascrizionalmente attiva di CLK, che potrebbe essere responsabile di una maggiore espressione dei geni *per* e *tim* e quindi dell'accumulazione delle rispettive proteine. Tuttavia, studi preliminari suggeriscono che la trascrizione non sia aumentata. Abbiamo analizzato la funzione di Usp5 in condizioni di alternanza luce:buio. In presenza dello stimolo luminoso, diversamente da quanto visto in condizioni di buio costante, le proteine CLK, PER e TIM subiscono una marcata, seppur parzialmente incompleta (se comparata alla condizione di un wild-type), degradazione. Questo suggerisce l'esistenza di meccanismi aggiuntivi (attivati dalla luce) capaci di regolare la degradazione circadiana delle proteine dell'orologio in condizioni di alternanza luce:buio. I dati finora ottenuti indicano per Usp5 un importante ruolo nella regolazione del feedback molecolare del clock auto-sostenuta dall'orologio stesso, vale a dire, attiva in condizione di buio costante.

# INTRODUCTION

One of the essential conditions for the surviving of an organism is its capacity to adapt to the environment. This includes the adaptation of its physiology and its behaviour to the cyclical daily and seasonal variations. Almost all organisms, from bacteria to mammals, have evolved with an internal "clock" capable of anticipating the periodic modification of the environment with suitable behavioural and physiological responses. In animals, circadian rhythms govern general physiology such as sleep-wake cycles, glucose, lipid and drug metabolism, heart state, stress and growth hormones secretion, temperature and immunity, as well as basic cellular process such as DNA repair and the timing of cell division cycle (Takahashi J.S. et al., 2008).

In humans, alteration of the clock timing has been associated with familial advanced sleep-phase syndrome, FASPS. This is an autosomal dominant behavioural disorder that causes early sleep time, early morning awakening and a short circadian period. Mutations in genes coding clock proteins (the kinase CKIdelta and its phosphorylation site on PER2) have been identified in families with FASPS. Opposite to FASPS, DSPS (delayed sleep-phase syndrome) causes late sleep onset and the inability to wake up at a conventional time. Individuals with FASPS fall asleep several hours before nightfall and waken before sunrise; whereas individuals affected by DSPS are well active into the night and sleep for hours after sunrise. Since then, multiple general illness have been linked to the un-synchronization of the endogenous clock to human lifestyles. The disruption of a circadian rhythm, as experienced by people subjected to jetlag or night shift-work, causes significant physiologic stress and predisposes individuals to insomnia, depression, obesity and cancer (Gallego M. and Virshup D. M., 2007).

All the circadian clocks share the same general properties. By definition, biological clocks keep functioning in the absence of external stimuli (hence, in constant conditions), but they are entrained by environmental cues, called

zeitgebers (from the German, “time-giver”, synchronizer), the most important of which are light and temperature. Different from general biochemical reactions, which are very sensitive to temperature, clocks are temperature-compensated, hence their period is almost independent from temperature.

*Drosophila* has been a powerful model to understand the molecular clock machinery. *Drosophila* genetics have provided the field of circadian rhythms with most of the known clock genes, which turn out to be highly conserved between flies and mammals, including humans (although species-specific roles exist for some of them). Deciphering the molecular bases of *Drosophila*’s clock could thus be relevant to better understand the human circadian system.

## Rhythmic behaviours in *Drosophila melanogaster*

*Drosophila* is a remarkable organism to study circadian rhythms. Eclosion (emergence of the adult flies out of the pupal case) was initially used to genetically dissect circadian rhythms, but the most used circadian output is currently locomotor activity (Dubruille R. and Emery P., 2008).

In laboratories, the flies are entrained to daily cycles of 12 hours light: 12 hours dark (LD 12:12). Conventionally, we refer to ZT0 (zeitgeber time) as the time of lights-on and ZT12 as the time of lights-off. In these conditions, *Drosophila* flies are bimodal and “crepuscular”, displaying a bout of activity around lights-off to lights-on transition (referred as the morning peak), and then, after a “siesta” in the mid-afternoon, a second more prominent burst of activity around lights-on to lights-off transition (the so-called evening peak) (**Fig.1**). Males show a narrower distribution of activity around the times of light transition and a more marked “siesta” in the middle of the day (Rosato E. and Kyriacou C.P., 2006). Importantly, as for all diurnal animals, light promotes activity. This direct effect of light is independent of the clock (“masking effect” of light is present also in flies without a functional circadian clock, and disappear in constant darkness) but can conceal the locomotor activity controlled by the clock (Rieger D. et al., 2003). The clock functioning is thus shown by the ability to anticipate transitions: the

activity increases before lights-on and lights-off.

Once put in constant darkness conditions (DD), each fly free-runs according to its own internal clock period. In this case flies are unimodal: 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). During constant darkness conditions, time is only given by the internal clock and thus we refer at it as circadian time (CT).

In constant light conditions (LL) wild-type flies are completely arrhythmic (AR) (see below, *Synchronization of the clock by light*).

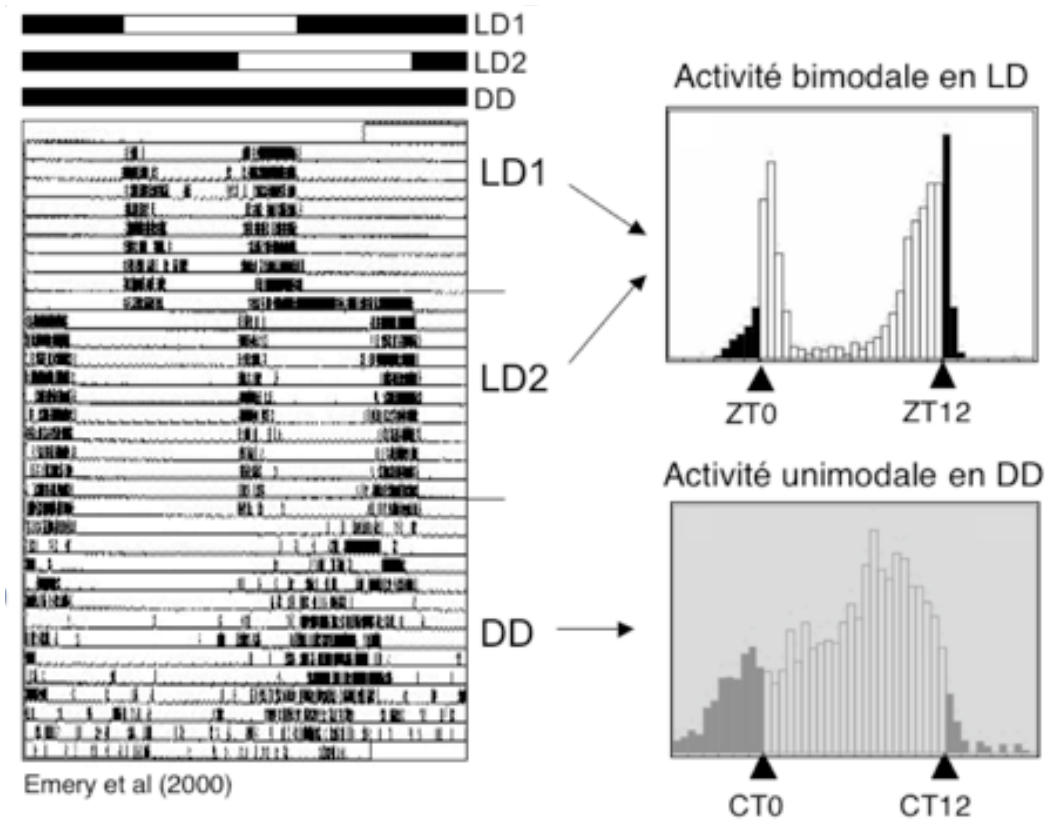


**Fig.1** Locomotor activity of *Drosophila melanogaster*. The fruit fly display a bimodal activity in LD conditions and an unimodal activity in DD conditions. Actograms (on the left): the flies rapidly synchronize after a shift of the light:dark phase (between LD1 and LD2). Day and night times are shown on the top by white and black boxes, respectively. Every black bar in the actogram represents a number of the times the fly has broken the infrared beam (from Emery P. et al., 2000). Eduction (on the right): each box in the histogram represents the average locomotor activity of the fly during 30 min (bin). ZT0 and ZT12 mean, respectively, the time of the lights-on and the time of the lights-off. CT0 and CT12 mean the times of the lights-on and off during the entrainment before the constant darkness conditions.

## Localization of the *Drosophila* clock(s)

It has been shown that individual cells throughout the fruit fly body do possess an intrinsic and autonomous clock that ticks in the absence of external stimuli and is capable of light:dark cycles-driven entrainment. In *Drosophila* as in other animals, we can distinguish a brain clock and peripheral clocks. The brain clock controls directly general complex behaviours like the sleep-wake rhythm. Peripheral clocks function in a mostly independent manner and can tick according to their own specific phase.

The *Drosophila* brain clock includes almost 150 neurons, organized in different groups and named according to their anatomical localization (**Fig.2**). These pacemaker neurons are located in the lateral (lateral neurons, LNs) and dorsal (dorsal neurons, DNs) 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 ($LN_d$s), the large ventral LNs (l-$LN_v$s), the small ventral LNs (s-$LN_v$s), and the 5$^{th}$ s-$LN_v$. The s-$LN_v$s alone appear to be sufficient for locomotor rhythmic output in total darkness conditions (Grima B. et al., 2004; Nitabach M.N. and Taghert P.H., 2008), whereas $LN_d$s and the 5$^{th}$ s-$LN_v$ can drive free-running rhythms in LL (Picot M. et al., 2007). The l-$LN_v$s and the s-$LN_v$s, but not the $LN_d$s and the fifth s-$LN_v$ express the neuropeptide pigment-dispersing factor (PDF), which is required for the free running locomotor activity in DD (Nitabach M.N. and Taghert P.H., 2008). The l-$LN_v$s do not seem to free-run in constant darkness, and their circadian function is still unknown. The group of LNs located in the posterior brain is called LPN (lateral posterior neurons). The LPNs may play a special role in the synchronization to temperature cycles. The DNs consist of three main groups – the $DN_1$s, $DN_2$s and $DN_3$s. A subset of the $DN_1$s has been specifically implicated in driving oscillatory behaviours in constant light (LL) conditions (Murad A. et al., 2007; Stoleru D. et al., 2007).

As previously said, in LD conditions flies are bimodal, showing an increase in locomotor activity that anticipates lights-on and lights-off transitions. Separate oscillators control these morning and evening peaks (Grima B. et al., 2004; Stoleru D. et al., 2004). The morning activity peak is driven by the $LN_v$s, whereas the evening peak is controlled by the PDF-negative LNs and possibly a subset of

$DN_1$s. Communications among these oscillator neurons, possibly by PDF or some others neurotransmitters, may enhance the robustness and modulate the phase of locomotor activity rhythms.

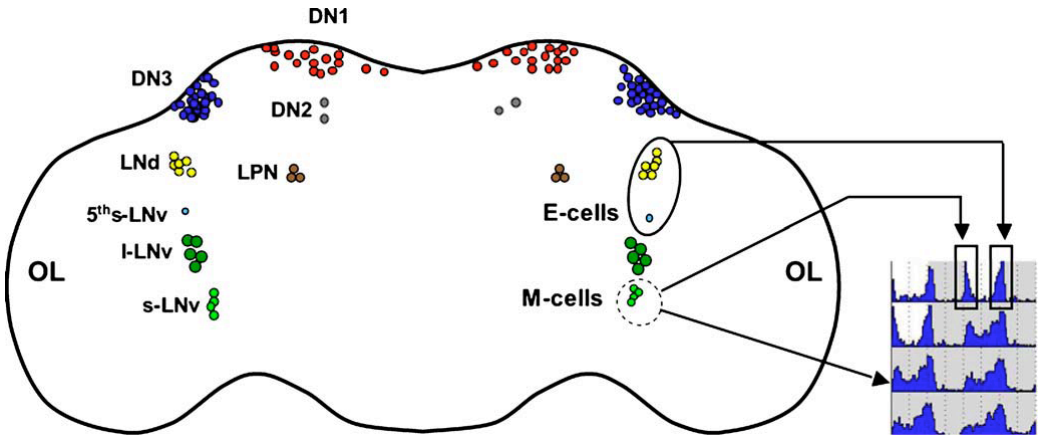


**Fig.2** The circadian clock neurons in the *Drosophila* brain. The $sLN_v$s are frequently referred as the "morning cells" (M-cells), since they are necessary for the morning activity peak under LD conditions. They are also required for free-running locomotor activity rhythms in DD. The fifth $sLN_v$ and the $LN_d$s control the evening activity under LD conditions and are therefore called "evening cells" (E-cells). OL means Optic Lobe. From Dubruille R. and Emery P., 2008.

## Molecular basis of the *Drosophila* clock

But what is *Drosophila* circadian rhythmicity made of? Circadian rhythmic behaviours and physiology are controlled by neuronal and probably hormonal signals induced as output of a molecular oscillator made by two main interconnected protein feedback loops, centered on a positive element: the transcription activator complex made by CLOCK (CLK) and CYCLE (CYC) transcription factors (**Fig.3**).

In the evening, CLK and CYC heterodimer induces the transcription of *period* (*per*) and *timless* (*tim*) genes. In the cytoplasm, PER and TIM proteins interact and then move (apparently separately) to the nucleus during the night, where they act as repressors of CLK/CYC transcriptional activators. Once in the nucleus, TIM is first degraded and then PER (which appears to be the main transcriptional repressor), allowing the CLK/CYC-dependent transcription to restart. The timing of PER and TIM accumulation is regulated post-translationally, introducing a delay compared to the accumulation of their mRNA (see *Post-translational modifications of clock proteins*). Other CLK/CYC activated genes feedback on the expression of *clk.* In this second feedback loop, the CLK/CYC heterodimer binds E-boxes to activate high level of VRILLE (VRI) and Par domain protein 1epsilon (PDP1epsilon) expression during the late day and early night. VRI accumulates in phase with its mRNA and binds to VRI/PDP1epsilon box (V/P box) regulatory elements to inhibit *clk* transcription. PDP1epsilon was thought to compete with VRI to V/P box binding in order to activate transcription, but its contribution to this feedback loop has been recently questioned (Benito J. et al., 2007). *Clk* and *cyc* non-functional mutants exhibit constant peak levels of CLK expression, suggesting that a clock-independent activator may drive constitutive *clk* transcription that is then rhythmically modulated by VRI (Hardin P.E., 2005).

Different *Drosophila*'s tissues share this general model: either the clock neurons, responsible of driving locomotor behaviours, or peripheral tissues, like eyes and antennae. Notably, these general features of the clock do show tissue-specific regulatory partners and modifications. Notably, the CRYPTOCHROME (CRY) photoreceptor appears to act as a clock protein in *Drosophila* peripheral tissues (Krishnan B. et al., 2001; Ivanchenko M. et al., 2001; Collins B. et al., 2006).

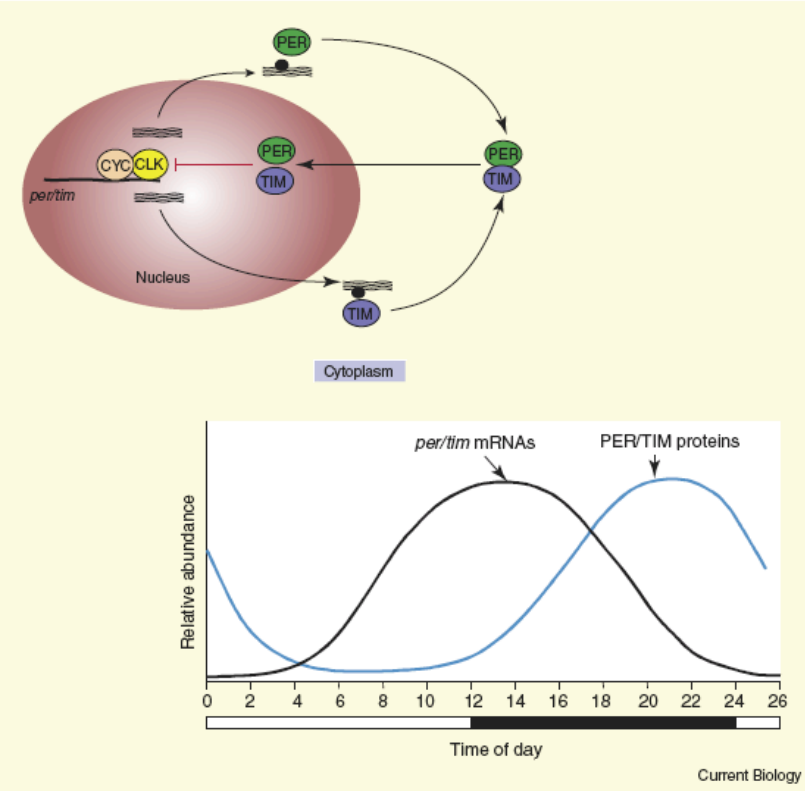


**Fig.3** *Drosophila* molecular circadian oscillator. Expression of the *per* and *tim* genes is promoted by the heterodimeric CLK/CYC transcription factors and reaches a peak late in the day. Translation of *per* and *tim* mRNAs lead to the gradual accumulation and dimerization of PER and TIM proteins in the cytoplasm. The protein levels peak in the night, during which time they enter the nucleus to inhibit CLK/CYC transcriptional activity. From Nitabach M. and Taghert P., 2008.

## Post-translational regulation of clock proteins

Post-transcriptional regulation is crucial to permit the delay in the feedback loop that is required to give the clock an approximate 24-hours period. If, following synthesis, the repressor proteins PER and TIM translocate directly to the nucleus to block CLK/CYC-mediated transcription, the whole cycle would take just a few hours rather than one day (Gallego M. and Virshup D.M., 2007).

Reversible phosphorylation plays a prominent role in the core oscillatory mechanism. Specific clock proteins are subjected to different phosphorylation programs that affect their activity, their stability and presumably their sub-cellular localization, finally setting period, amplitude and phase of the clock (**Fig.4**).

*De novo* synthesized PER is first phosphorylated in the cytoplasm, followed by

progressive increase in phosphorylation, that continue in the nucleus until the early day (Bae K. and Edery I., 2006). TIM binding limits but does not eliminate the increasing phosphorylation of PER, whose hyper-phosphorylated form is finally recognized by the F-box/WD40 protein SLIMB (SLMB), that target it to the 26S proteasome for destruction. DOUBLETIME and CASEIN KINASE II are the two best-characterized kinases acting on PER destabilization. Notably, because phosphorylation is reversible, protein kinases as well as protein phosphatases play important roles in determining the clock timing. PER phosphorylation level is also regulated by the activity of PROTEIN PHOSPHATASE 2a (PP2a), which stabilizes PER via dephosphorylation. In addition to determine PER stability, phosphorylation seems to determine even its localization. DBT-mediated phosphorylation seems to hold PER in the cytoplasm, whereas dephosphorylation allows its nuclear entrance. It also appears that phosphorylation plays a role in CLK function. There are several phospho-variants of the CLK protein, the highly phosphorylated ones seem to be less competent to drive transcriptional activation. DBT and PP2a appear to be important regulators not only for PER stability but also for maintaining CLK phosphorylation levels within a certain functional range. The nuclear entry of phosphorylated PER would induce CLK phosphorylation and its unbinding from DNA (Yu W. et al., 2006). Similarly to PER, TIM protein levels begin to raise in the early night and rapidly disappears prior to and especially following the onset of dawn. A critical TIM kinase is SHAGGY (SGG), a homolog of mammalian GSK3, which phosphorylates TIM to promote nuclear localization of PER/TIM heterodimers. TIM light-induced degradation is mediated by its binding to the blue-light photoreceptor CRYPTOCHROME and requires the F-box/LRR E3 ubiquitin ligase JETLAG (JET) (Koh K. et al., 2006; Peschel N. et al., 2006) (see below). As a circadian oscillation persists in constant darkness conditions (DD), a mechanism exists that controls TIM (and consequently PER) accumulation in the absence of light-induced CRY-mediated TIM destruction. The kinase CKII has been recently shown to mediate TIM cycling in DD (Meissner R.A. et al., 2008).

It is generally thought that the feedback timing during light:dark cycles or in free running DD conditions relies on different post-translational mechanisms, most importantly destruction. In constant darkness, highly phosphorylated forms of the

PERIOD and TIMELESS proteins are constitutively present in the F-box/WD40 protein *slmb* (that mediates ubiquitination and proteasome targeting) mutants, indicating that the control of their cyclic degradation is impaired (Grima B. et al., 2002; Ko H.W. et al., 2002). Because levels of PERIOD and TIMELESS oscillate when mutants are maintained in light:dark conditions, light- and clock-controlled degradation of PERIOD and TIMELESS do not rely on the same mechanisms (Grima B. et al., 2002; Koh K. et al., 2006).

Opposite to the notable role of post-translational modifications, circadianly controlled gene expression seems to be less critical for the function of the clock. The oscillations (PER and DBT-mediated) of CLK/CYC capability of PER promoter binding drive rhythmic expression of the *per* gene. However, flies in which PER is expressed from a constitutive rather a rhythmic promoter still have a normal rhythm (Yang Z. and Sehgal A., 2001). So, even if circadian outputs (such as sleep, metabolism and locomotor activity) are supposed to be controlled by changes in output genes transcription, the core clock that drives these changes in gene expression might be regulated mostly by post-transcriptional modifications. Although transcriptional control might be more important for TIM regulation (Yang Z. and Sehgal A., 2001), phosphorylation-regulated, ubiquitin-directed, proteasome-mediated proteolysis of clock proteins is crucial.

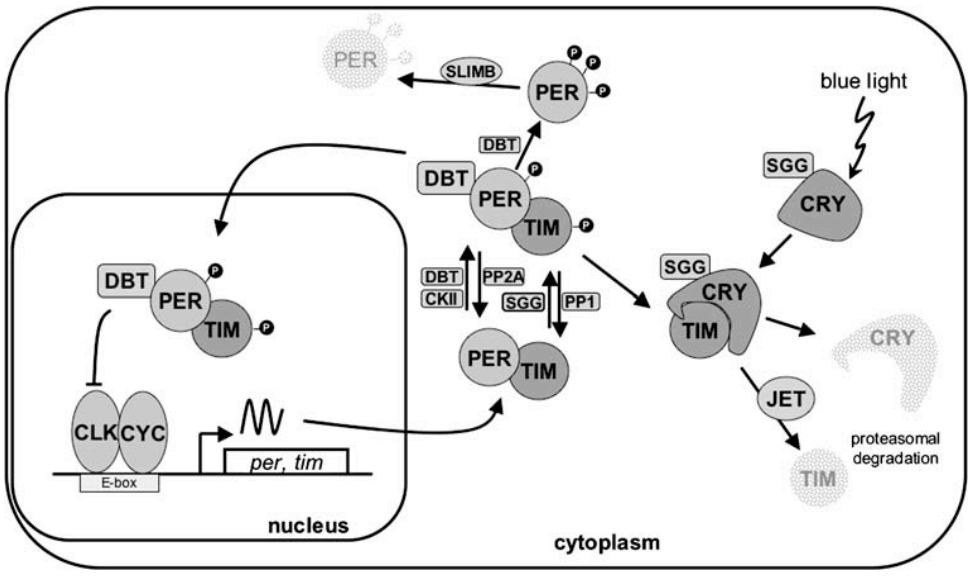


**Fig.4** The *Drosophila* circadian pacemaker and the CRY input pathway. See main text for details. Speckled shapes indicate proteins undergoing proteasomal degradation. P: phosphate groups. From Dubruille R., and Emery P., 2008.

## Ubiquitinylation and proteasome pathway of degradation

The proteasome plays a central role in the cell functioning. It controls the degradation of misfolded proteins, old or in excess, whose accumulation would be toxic. It is also involved in the degradation of cellular regulators and the maturation of polypeptidic precusors, as well as the production of peptidic antigenes. It is thus involved in the most important functions and cellular decisions, such as cellular division, differentiation, apoptosis, external signals transducing pathway and adaptative immunoresponses (Andermarcher E. et al., 2005).

Ubiquitin (Ub) is a small conserved ubiquitous polypeptide made by 76 aminoacides. It is synthesized as peptide-linked ubiquitin polymers or ubiquitin-protein fusions. Ubiquitin is conjugated to proteins by an isopeptidic bound between its terminal glicine (GLY76) and an internal Lys of the substrate. The ubiquitin itself possesses seven lysines (K6, K11, K27, K29, K33, K48, K63), each of which lets auto-polymerization that forms poly-Ub chains. Ub-linking to proteins could vary in position, length and branching of the ubiquitin chains. This variety permits different protein modifications, thus allowing ubiquitin to be involved in multiple signalling functions other than targeting proteins for degradation (Andermarcher E. et al., 2005).

Although there are now evidences that a lot of proteins do not or do not always require ubiquitination to be recognized by the proteasome, proteins targeted for elimination are generally ubiquitinated. They undergo a post-translational modification, like phosphorylation, which favors the covalent linking of a poly-Ub chain. Protein ubiquitination requires three different enzymes: E1, E2 and E3. E1 (Uba) is an Ub-activating enzyme, unique, which activates ubiquitin and transfers it to the E2 enzyme (Ubc). This Ub-conjugating enzyme directly, or helped by the E3 enzyme, transfers and polymerizes ubiquitin monomers on the substrate. Thanks to their high number, the E3 ubiquitin-ligase enzymes carry the specificity of the reaction. The best-known E3 complex involved in circadian rhythms in *Drosophila* is the SCF-E3 ubiquitin ligase. It consists of the linker protein Skp1, the scaffold protein Cul1 – which recruits the E2 Ub-conjugating enzyme, and an F-box protein that recruits phosphorylated substrates. The F-box

proteins SLMB and JET are central components of *Drosophila* circadian clock. $SCF^{Slimb}$ induces DBT-phosphorylated PER ubiquitination and proteasome degradation, and regulates TIM stability in DD. $SCF^{JET}$ controls TIM and CRY degradation during LD cycles. SCF complexes are generally activated to ubiquitinate substrates by covalent attachment of Nedd8, an ubiquitin-like modifier, to Cul1, and inactivated by the deneddylase activity of the COP9 signalosome (CSN) subunit 5 (CSN5), which has been very recently shown to be involved in the *Drosophila* clock (Knowles A. et al., 2009).

## Deubiquitinating enzymes

Ubiquitination is reversible. The de-ubiquitination process is catalyzed by a large class of specialized proteases called deubiquitinating enzymes (DUBs). DUBs maintain optimal levels of cellular ubiquitin (which is otherwise produced at a relatively low level) by recycling Ub attached to inappropriate targets, removing and disassembling poly-Ub chains, and preprocessing proteins degraded by the proteasome (Amerik A.Y. and Hochstrasser M., 2004). Moreover, DUBs are responsible for processing inactive ubiquitin precursors, and for keeping the 26S proteasome free of unanchored ubiquitin chains that can compete with ubiquitinated substrates for ubiquitin-binding sites. The DUBs family comprises at least five distinct subfamilies based on sequence similarity and putative mechanisms of action. The two main classes of DUBs are ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (USPs/UBPs). UCHs are relatively small enzymes (20-30 kDa) that catalyze the removal of peptides and small molecules from the C-terminus of ubiquitin. USPs varies greatly in size (50-300 kDa) usually with N-terminal extensions, which may function in substrate recognition, subcellular localization and protein-protein interactions. USPs can process ubiquitin precursors, remove ubiquitin from protein conjugates and disassemble ubiquitin chains (Amerik A.Y. and Hochstrasser M., 2004). DUBs are characterized by the presence of three conserved domains surrounding, respectively, one cysteine (Cys box), one aspartic acid (Asp box) and two histidine residues (His box), which form the catalytic

triad.

The amount of free ubiquitin and free polyubiquitin chains is very important in a cell. Free polyubiquitin chains can link to the 26S proteasome at its ubiquitin-recognition signal and actively compete to polyubiquitin-linked substrates tagged for proteasome degradation. Polyubiquitin chains can be generated endogenously by ubiquitin-ligating enzymes or through release from poly-ubiquitinated substrates by DUBs. Surprisingly, a single DUB is responsible for the bulk of unanchored poly-ubiquitin chains in vivo, at least in yeast. This DUB, called Ubp14 in yeast and USP5/IsopeptidaseT in mammals, specifically disassembles ubiquitin oligomers from the proximal end, i.e. the end with an un-attached Gly76 carboxyl group. This enzyme is very sensitive to any modifications to the C-terminus of the proximal ubiquitin in the chain, which is exceptionally recognized by a deep pocket in the enzyme. This prevents Ubp14/IsoT from acting on polyubiquitinated protein substrates and restricts its function only to unanchored polyUb chains (Amerik A.Y. et al., 1997).

The *Drosophila* genome contains at least 18 (probably 19) UBPs (Chen X. and Fischer J.A., 2000). All of them share eight blocks of sequence conservation within the catalytic region, including the Cys and His domains. Fat facetes (Faf) is the best-characterized UBP in *Drosophila*. This large deubiquitinating enzyme has shown to be required for the pattering of the fly eye and for the cellularization of the embryo.

## Synchronization of the clock by light

Light is the most important zeitgeber acting to synchronize the endogenous clock to daily cycles. When the light:dark cycle is shifted, wild-type flies quickly (about one day) adjust their locomotor activity rhythms according to the new phase. To understand how light is able to entrain the clock, one can study how the clock responds to a pulse of light that is given at different times of the cycle during DD conditions. Light application during the subjective day (i.e. the period in which light would normally be present in LD) yields no or little phase change, whereas a delay and advance of phase are shown when the pulse is given in the early and late night, respectively (phase response curve, PRC). If the pulse is given during the early night, it is perceived as a late sunset and the clock will be delayed accordingly. *Vice versa* light applied during the late night is felt as an early dawn and the clock will respond with an advance of the phase.

Light entrains the circadian oscillator by activating a photoreceptor that directly or indirectly alters the level or activity of an oscillator component (Hardin P.E., 2005). The light-mediated degradation of TIMLESS is considered as the initial clock-specific event in the entrainment of the clock to light-dark cycles. This light-dependent degradation may produce phase advances and delays depending on the level of *tim* mRNA. Early in the dark phase, TIM levels could rebound after light-induced degradation thanks to the elevated level of *tim* mRNA, but the delay in TIM accumulation determines a phase shift. Late in the dark phase, TIM levels could not rebound after light-induced degradation because of the low levels of *tim* mRNA, fast forwarding to the next phase of the circadian cycle and advancing the phase of the clock. During the light-time, TIM interacts with CRYPTOCHROME, which is considered to be the most important light mediator to the clock. As *cry* mutant flies can still be entrained by light, other signals from the visual system can reset the brain clock in a CRY-independent manner (Emery P. et al., 2000). *Drosophila* possesses three main photoreceptors and/or photopigments that appear to be involved in the circadian photoreception (**Fig.5**): (1) the compound eyes, (2) the Hofbauer-Buchner eyelet and (3) the blue-light photoreceptor Cryptochrome. The different contribution of each of them for the synchronization to light is not yet well established. The photoreceptive pathway of

*Drosophila* visual system uses rhodopsins as photoreceptors. The outer photoreceptors of the retina, R1-6 cells, express a single opsin, Rh1. They are the most sensitive detectors of visible light and are critical to the fly's ability to perceive motion. The inner retinal photoreceptors, R7 and R8, express different combinations of rhodopsin. Photoreceptors R7 shows rhodopsin Rh3 and/or Rh4, while photoreceptors R8 express Rh5 and Rh6. Rhodopsin Rh6 is also expressed in the Hofbauer-Buchner eyelet, a sub-retinal structure that sends axons to the PDF-expressing clock neurons. The rhodopsin-mediated phototransduction cascade involves an eye-specific Phospholipase C (PLC) encoded by the *norpA* gene. PLC acts in both larval and adults photoreceptors and operates through a partway independent of CRY (Stanewsky R. et al., 1998; Ivanchenko M. et al., 2001).

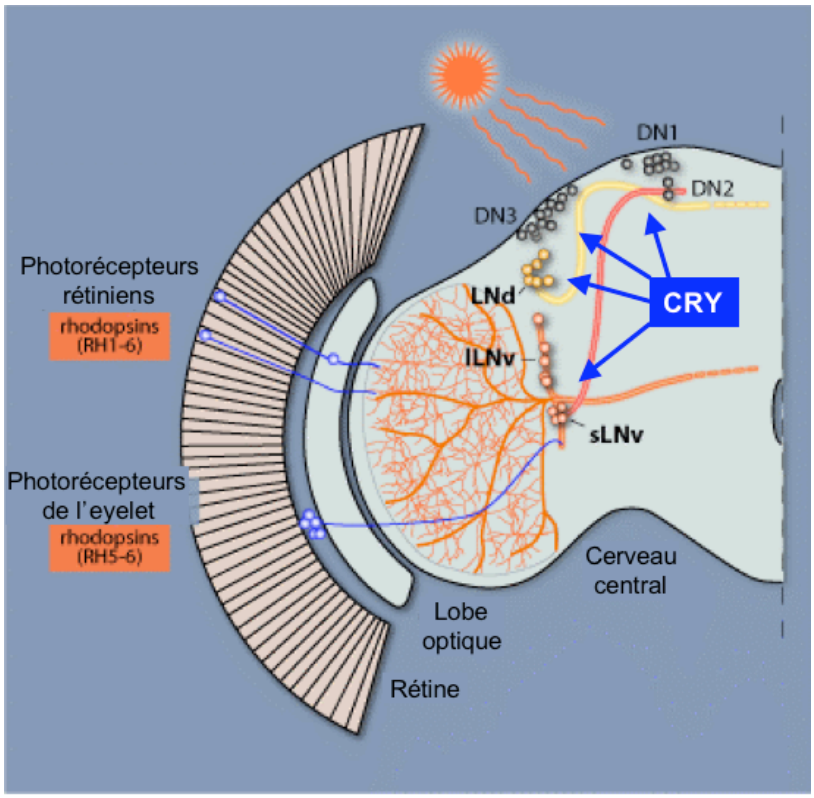


**Fig.5** Circadian photoreception systems of the *Drosophila* brain clock: the retinal photoreceptors (Rhodopsins 1-6), the photoreceptors of the Hofbauer-Buchner eyelet (Rh5-6) that contact the $LN_vs$, and Cryptochrome (CRY), directly expressed in the clock neurons. From Helfrich-Forster C. et al., 2004.

## *Drosophila* CRYPTOCHROME

Cryptochromes are a class of flavoprotein blue-light signaling receptors found in plants and animals (Hoang N. et al., 2008). They exhibit high sequence and structural similarity to the light-dependent DNA-repair enzyme photolyase, but have lost the ability to repair DNA. Indeed, they use the energy from the near UV/blue-light to regulate a variety of growth and adaptative processes.

*Drosophila* CRYPTOCHROME contains a conserved N-terminal photolyase domain, responsible of the light-mediated activation, and a unique very short carboxy-terminal domain. It is thought that, when stimulated by light, CRY undergoes a conformational change that eliminates the repression maintained by the C-terminal domain to the ammino-terminal one. In fact, mutants lacking the carboxy-terminal domain are constitutively active (Dissel S. et al., 2004; Hemsley M.J. et al., 2007). Activated CRY is then able to interact with TIM (**Fig.6**) and recruit the F-box protein JET. $SCF^{Jet}$-E3 Ubiquitin ligase complex then mediates TIM polyubiquitinylation and its subsequent degradation by the proteasome. Light also promotes CRY's proteasome degradation, even if more slowly than TIM, underlying the light-dependent rhythm in CRY levels (Hardin P.E., 2005). Even if CRY and TIM degradation processes are supposed to be independent, there are evidences for CRY, TIM and JET to function in the same molecular pathway (Peschel N. et al., 2006; Peschel N. et al., 2009). It has been shown that CRY may be phosphorylated by the kinase that controls TIM phosphorylation and nuclear entry, SHAGGY (SGG) (Stoleru D. et al., 2007).

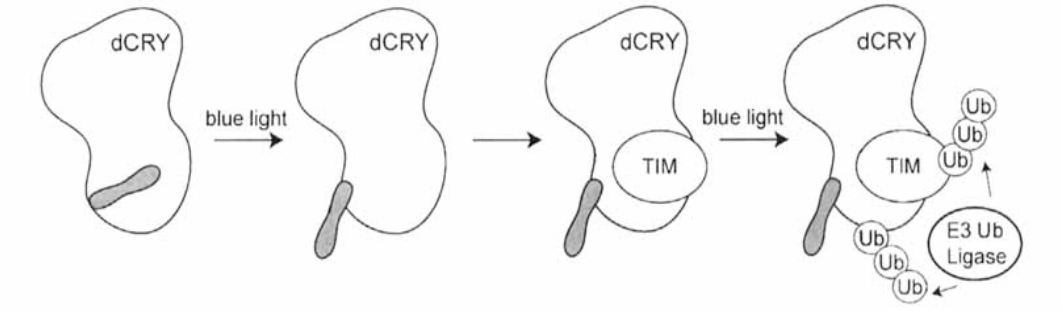


**Fig.6** Model of *Drosophila*'s CRY phototransduction. Irradiation of CRY with light relies the short C-terminal domain from the photolyase-homology region, allowing TIM to bind. Moreover, CRY induces TIM (and itself) for ubiquitin-mediated degradation, by a mechanism that involves JET of the SCF-E3 Ubiquitin-ligase complex (From Partch C.L. and Sancar A., 2005).

Likely in response to the continuous CRY-mediated TIM destruction, flies subjected to a regime of constant light for more than two days are arrhythmic, i.e. they lose the periodicity of locomotor activity behaviour. On the contrary, flies mutant for *cryptochrome* can retain rhythmicity in LL and are insensitive to short light pulses. The mutant $cry^{baby}$ ($cry^{b}$) (Stanewsky R. et al., 1998) possesses a non-sense mutation in the flavine domain that gives rise to a strong hypomorphic – if not null, CRY protein. These flies can still be entrained by LD cycles, but their response to phase-shift are slower, hence, as opposite to wild-type flies, $cry^{b}$ mutants need more days to adjust to the new phase (Emery P. et al., 2000). Recently obtained null *cry* mutants ($cry^{0}$) behave similarly (Yoshii T. et a., 2008; Dolezelova E. et al., 2007). All these effects can be directly linked to the absence of light-mediated TIM degradation. It has been shown that rhythmicity in LL conditions is also achieved when two specific *timeless* and *jetlag* alleles are combined. Peschel N. et al. (2006) found that presumed WT *Veela* flies are rhythmic in constant light. This unexpected rhythmic behaviour in LL is due to the concomitant presence of the *timeless* allele $tim^{ls}$, which encodes a less light-sensitive form of TIMELESS, in combination with a mutant (natural) variant of the F-box protein JETLAG, called $jet^{c}$. Importantly, each of these variants is not able to induce abnormal locomotor behaviour in LL when carried alone.

*Cryptochrome* expression is under CLK/CYC transcriptional activation and occurs not only in the brain, where is located in most but not all (Benito J. et al., 2008; Yoshii T. et al., 2008; Zheng X. and Shegal A., 2008) clock neurons, but also in other body cells, giving them photosensibility. CRYPTOCHROME seems also to play an important light-independent role in peripheral tissues, where it appears to be part of the main feedback loop, as it is required for free-running activity rhythms (Ivanchenko M. et al., 2001). Here, CRY is thought to act as a transcriptional repressor, a role similar to the one played by the mammalian homologs mCRY1 and mCRY2 (Collins B. et al., 2006).

# PROJECT

The clock relies on two main interconnected feedback loops and the duration of the cycle depends on the post-translational modifications of the involved proteins. These regulations influence protein activity, localization and half-life, thus adjusting the phase and amplitude of the cycle to the environmental conditions. However we still do not know how the 24h pace of the molecular clock is defined or how CRY and signals from the visual system precisely affect the phase of the clock. This fine-tuning certainly relies on the activity of multiple regulatory proteins, most of which are not yet known. My project aims at discovering new clock partners, using two different strategies. On one hand we wanted to better define the activity of the main light-input transducer to the brain clock, CRYPTOCHROME. On the other hand we tried to understand the role of an ubiquitin protease, USP5, whose absence induces a lengthening of the clock period.

## CRY partners

Even if we have strong evidence for an important CRY role in the synchronization of clock by light, not much is known about the precise molecular steps involved in its phototransduction activity. Isolating the proteins that interact with CRY can help to understand this pathway.

**Table1** List of CRY putative partner proteins. Biological activity and functions are taken from Flybase. Domain description is inferred from a bioinformatic analysis using Prosite and ELM servers.

| CG number | Synonym | Domains and predicted activity / Annotations |
|---|---|---|
| CG31149 | Sperm-adhesin | CUB domain, LDL receptor domain. Involved in fertilization and development (many hypothetical functions). |
| CG4696 | Mp20 | Calcium ion binding; actin binding. Flight muscle-specific protein |
| CG5390 | | Ser-type endopeptidase activity. Proteolysis. |
| CG31795 | Ia2 | Protein Tyr-phosphatase activity; zinc ion binding. |
| CG4550 | NinaE (Rh1) | Opsin (phototransduction). Its mutation cause retinal degeneration, providing congenital night blindness. |
| CG7470 | | Takes part in the proline biosynthetic process. Circadianly controlled both in LD and DD (Ueda et al., 2002) |
| CG4662 | | Calcium ion binding. |
| CG2985 | Yp1 | Lipase. Structural molecule. |
| CG5939 | Prm | Myosin-tail1 domain. |
| CG8663 | Nrv3 | Sodium:potassium-exchanging ATPase activity. |
| CG4264 | Hsc70-4 | ATPase activity; unfolded protein binding; chaperone binding. Seems involved in axon guidance and nervous system development, neurotransmitters secretion.<br>Adult transgenic *timgal4*/UASRNAi flies died within few days. |
| CG17725 | Pepck | Phosphoenolpyruvate carboxykinase (GTP) activity. |
| CG17489 | RpL5 | Trans-2-enoyl-CoA reductase (NADPH) activity; zinc ion binding. Involved in fatty acid metabolic process; redox reactions. Embryonic lethal phenotype with *timgal4* driver. |
| CG12317 | Jhl-21 | Aminoacid transmembrane transporter activity. |
| CG10067 | Act57B | Structural constituent of the cytoskeleton. ATP and protein binding activity. |
| CG8804 | Wun | Lipid phosphate phosphatase (LPP). Proposed a role in the migration and viability of pole cells. Phosphatidic acid phosphatises (PAP) functions in opposition to diacilglycerol kinases (DGK, as RdgA in *Drosophila*), which take part in the phototransduction cascade. |
| CG10596 | Msr-110 | Unknown. |
| CG11909 | | Alpha-glucosidase activity. Carbohydrate metabolic process. |
| CG31689 | | AAA ATPase domain acting through the energy-dependent unfolding of macromolecules. ATPase activity coupled to transmembrane movement of substances. |
| CG6151 | | Unknown. |
| CG8588 | Pst | Involved in olfactory learning. |
| CG6806 | Lsp2 | Larval storage proteins are proteins from the hemolymph, which may serve as a store of aminoacids for synthesis of adult proteins. |
| CG34418 | Sif | Large protein of numerous domains, including Rho-GEF activity. Involved in Rho protein signal transduction. |
| CG2259 | Gclc | Glutathione biosynthetic process. |
| CG16935 | | Tans-2-enoyl-CoA reductase (NADPH) activity; zinc ion binding. It is involved in fatty-acid metabolic process; redox activity. |
| CG5125 | NinaC | Ser/Thr protein kinase. |
| CG3612 | Bellwether | ATPase activity (combine ATP synthesis and/or hydrolysis with the transport of protons across membrane). Pupal lethal with *timgal4* driver. |
| CG7998 | | L-malate dehydrogenase activity. Glycolysis. |
| CG8824 | Fused lobes | N-acetylglucosaminidase involved in N-glycan processing. |
| CG3874 | Fringe connection | Transmembrane nucleotide-sugar transport activity. Involved in embryonic morphogenesis. |
| CG2224 | | JAB_MPN domain involved in binding protein and proteasomal subunits. Mov34 domain that acts as regulatory subunit of the 26S proteasome, which is involved the ATP-dependent degradation of ubiquitinated proteins. |
| CG32031 | ArgK | Catalyze the transfer of phosphate from ATP to arginine. Phosphorylation. |
| CG4757 | | Carboxylestease activity, subfamily of Juvenile Hormone esterase. |
| CG1905 | | Unknown. |
| CG1066 | Shab | Voltage-gated potassium channel activity; protein binding (BTB domain). |
| CG30437 | | Cu-oxidase domain. Redox activity. |
| CG4000 | | Protein of the eggshell. |
| CG3805 | | Unknown. |
| CG31628 | Ade3 | *de novo* IMP biosynthetic process; purine base biosynthetic process. |
| CG10106 | Tsp42Ee | Tetraspanins are known to organize other protein (and/or enzymes) into a network of multimolecular membrane microdomains. |
| CG10966 | RdgA | Diacilglycerol kinase that phosphorylate glycerol to yeld phosphatidic acid. Involved in sensory perception of light stimulus. |

A recent paper (Sathyanarayanan S. et al., 2008) started investigating putative genes involved in CRYPTOCHROME stability. An assay that measures light-induced CRY-luciferase fusion protein degradation in cell culture was used to isolate genes through a *Drosophila* genome-wide RNAi screen. They found a large range of different presumed CRY interactors, including ubiquitin ligases, signal transduction molecules and redox molecules. Costa and co-workers in Padua adopted a different approach. Starting from a fly head cDNA library, they performed a yeast two-hybrid screen to detect any type of CRY-protein interaction. Moreover, they set up a co-immunoprecipitation assay with CRY-HA fusion proteins, and identified a number of interacting proteins using mass spectrometry analysis of the CRY-containing complexes. The result of these experiments is a list of more than 40 putative CRY partners, covering a wide range of predicted cellular functions (**Table1**). To test for a possible role of these candidates in regulating CRY function as a circadian photoreceptor, each of these genes was inhibited using a *UAS-RNAi* construct driven by a *tim-GAL4* driver, which is known to be expressed in all clock cells (in addition to other cells). The LL rhythmic phenotype of the *cry* mutants was used to assay RNAi-expressing flies for the involvement of the down-regulated gene in the CRY-dependent light input pathway.

## USP5

A major problem in the search for new clock genes relies in the fact that many of them could be required during development, making adult mutant flies difficult to isolate. The GAL4-UAS system represents a good tool to modify the expression of genes whose strong mutation would be lethal, and it can be used to specifically direct the expression of RNAi transgenes to clock neurons. In collaboration with the NIG-FLY (Japan) stock center, our laboratory has begun a *Drosophila* genome-wide RNAi screening project to search for new clock genes (see *Materials and Methods* for details). As a today, over 3000 *Drosophila* genes have been screened and 15 positives have been isolated.

One of the strongest phenotype observed was with the candidate gene *CG12082*. The *CG12082* gene encodes the ubiquitin-specific protease 5 (USP5), whose activity has not yet been characterized in *Drosophila*. As inferred from bioinformatic analysis (**Fig.7**), USP5 possesses four putative ubiquitin binding domains: the catalytic UBP (ubiquitin-specific processing protease) domain, formed by non-contiguous regions and containing the active-site Cys, Asp and His boxes, two ubiquitin-associated (UBA) domains, and a zinc-finger ubiquitin binding domain (ZnF UBP), also known as polyubiquitin-associated zinc-finger (PAZ) domain. The UBP-type zinc finger (ZnF UBP) is only found in a small subfamily of ubiquitin hydrolases, capped by human Isopeptidase T.


**Fig.7** Schematic representation of the conserved domains of *Drosophila* CG12082 protein inferred from NCBI-Entrez database. The UBP (Peptidase C19 superfamily) domain drives catalytic activity, while UBA1, UBA2 and the ZnF UBP (PAZ) are putative ubiquitin-binding domains.

Earlier behavioural and molecular studies on USP5 defective flies have been performed by Béatrice Martin and Sylvina Bouleau, under whose precious guidance I've continued the analysis of USP5.

Tested for locomotor behaviour in free-running DD conditions, flies expressing a double copy of the *UAS:usp5-RNAi* transgene in the $LN_vs$ (*gal1118* driver, see *Materials and Methods*) display a strong period lengthening of about 5-6 hours (tau= 29-30h). To confirm that the phenotype is specifically caused by Usp5 down-expression, two other transgenic *usp5-RNAi Drosophila* lines - from the VDRC stock center (Vienna) – were tested, leading to the same long-period phenotype (**Table2**). To support specific *usp5* gene targeting by the RNAi, RT-PCR in fly heads was performed and showed a decrease of CG12082 mRNA levels (data not shown). Moreover, a P-element inserted at the very end of the gene exists (mutant #22426, from the Bloomington stock center) but homozygous flies are lethal and the heterozygous mutation does not induce an altered period.

To analyze the molecular defects induced by the *usp5* RNAi, PER and TIM levels in the s-$LN_v$s were quantified in whole-mounted brains. PER and TIM oscillations in adult fly brains well reproduce the long-period phenotype shown by behavioural analysis (see *Results*). The more ubiquitous *timgal4* driver was then used to analyze PER and TIM proteins by western blots of fly heads protein extracts. In DD conditions, these flies display only 1.5h period lengthening. This is very likely due to the weaker *timgal4* expression (compared to *gal1118*) in the s-$LN_v$s (which drive activity rhythms in constant darkness). In constant DD conditions, both brain and head extract experiments show that PER, TIM and CLK proteins persist abnormally during the all day, indicating abnormal accumulation and/or absence of degradation (see *Results*).

| Genotype | Alive flies (tested) | % R | Tau (sem) | Power (sem) |
|---|---|---|---|---|
| w;12082$^{NIG}$;*gal1118*/+ | 17 (17) | 94,1% | 29,6 (0,37) | 89,4 (9,5) |
| w;;*gal1118*/+ | 12 (14) | 91,7% | 23,9 (0,06) | 214,9 (14,62) |
| w;12082$^{VDRC}$;*gal1118* | 12 (16) | 100,0% | 27,7 (0,22) | 141,1 (10,04) |
| w;;*gal1118* | 15 (16) | 100,0% | 24,4 (0,08) | 145,0 (10.94) |
| w;12082$^{NIG}$/*timgal4*;12082$^{NIG}$/+ | 22 (24) | 95,5% | 26,5 (0,27) | 127,6 (12,58) |
| w;*timgal4*/+ | 16 (16) | 100,0% | 24,9 (0,18) | 186,2 (15,96) |

**Table2** Locomotor activity data from *usp5-RNAi* expressing flies. The RNAi expression is induced by *timgal4* or *gal1118*-GAL4 drivers, whose only insertion is taken as control. The first column defines the genotype tested (see *Materials and Methods* for nomenclature). The second column shows the number of flies that survive until the end of the test and, on brackets, the number of flies that was originally put on monitors. The rhythmicity column shows the percentage of rhythmic flies among those that success to arrive at the end of the experiment. Then is reported the period length (tau) and its standard deviation (sem). Finally the power (with its sem), which indicates the robustness of the rhythmic phenotype displayed.

These data introduce some obvious questions:

- The long-period phenotype is a reproducible characteristic of *usp5-RNAi* flies taken in DD free-running conditions. Do transgenic flies still display an altered phenotype when the light re-setting stimulus is present? To answer, *RNAi*-carrying flies will be tested for locomotor activity both in LD and LL conditions.
- The long-period locomotor phenotype is sustained by lengthened oscillations of PER and TIM proteins. How do these proteins cycle in LD conditions? Adult brain immunolabeling and western blot analysis with total head extracts of flied entrained by 12h:12h light-dark cycles will be done.
- Larvae homozygous for the P-element insertional mutation in the *usp5* gene fail to enter the pupal stage and died. Do 3$^{rd}$ instar (the stage before the pupa) larvae possess morphologically normal clock neurons? And do they display an altered clock protein cycling in free-running DD conditions? Larval brain dissections will allow to answer this.
- In order to better visualize the molecular effect of Usp5 down-expression we have to find the conditions that give the strongest altered phenotype. When driven by *timgal4*, RNAi expression leads to a slightly lengthened period of locomotor activity (tau= 25,5h). To increase the RNAi expression, we will produce a driver line carrying the *timgal4* transgene together with the *UAS-dicer2* insertion, which is known to increase the RNA interference action.

# MATERIALS AND METHODS

## Nomenclature in *Drosophila*

The fly genome contains about 15000 genes, scattered on 4 pair of chromosomes. The information carried by the couple of chromosomes 1 defines the sex of the fly (sexual chromosomes). The fourth chromosome is very small and generally not studied.

Some rules have been established to define a fly's genotype:

- Semi-colons separate the genotype symbols for different chromosomes, and chromosomes are always listed in the order: X/Y ; II ; III ; (IV)
- For each chromosome, the genotype is indicated only if there's a mutation. When the genotype is not written, or a plus (+) compare, the chromosome must be intended wild-type.
- A chromosomal genotype written on a single line indicates that the stock is homozygous for that genotype. Heterozigosity is denoted by a two-lines genotype (the genotype for each homologue is separated by "/").

## Maintenance of *Drosophila*'s strains

Flies were raised on standard cornmeal (flour of maize, yeast and agar) medium in 12h light:12h dark cycles (LD). At 19°C temperature a *Drosophila* complete life cycle lasts about 20 days. Quicker (10 days) eggs to adulthood cycle is obtained when putting fly tubes in incubators at 25°C.

## Behavioural analysis

*Drosophila* locomotor activity rhythms have been analysed as in Klarsfeld A. et al., 2003. Briefly, individual male flies 1-3 days old were inserted in small glass tubes half filled with sucrose-containing gelose and loaded in 32 channels activity monitors (as shown in **Fig.8A**). The monitors were then housed in a light- and temperature-controlled incubator. Each channel in the monitor is equipped with infrared emitters and receivers associated to a computer, which count every time the IR beam is broken during a defined (30 minutes) period of time (bin) (**Fig.8B**). The recording for each bin up to 24 hours give an indication of the daily pattern of activity and rest of the fly. Locomotor activity could be displayed for multiple days one next-to the other, giving rise to a plot called *actogram*. This visualization permits to easily observe periods longer or shorter than the canonical 24 hours length. Plotting the average CT/ZT bin for each day tested on a 24-hours histogram shows morning and evening anticipatory activity peaks (*eduction*).

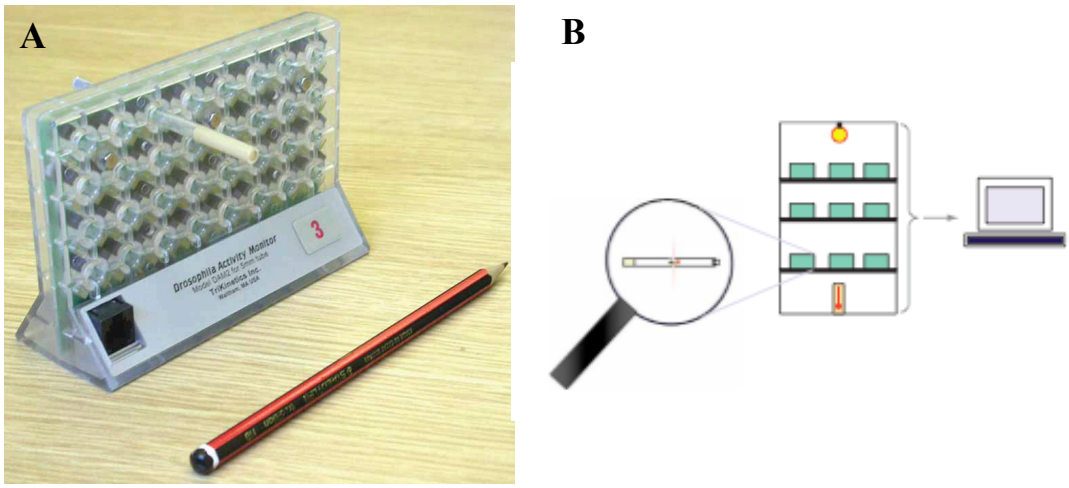


**Fig.8** **A** *Drosophila* activity monitors from TriKinetics: a single DAM2 monitor that will take 32 individual activity tubes. **B** Setup for locomotor activity analysis. Individual flies are inserted in small glass tubes. These are loaded in activity monitors (green boxes) housed in a light- and temperature-controlled incubator and connected to a computer. The activity monitors contain the infrared emitters and receivers and the associated computer components. When a fly breaks the infrared beam across its path, the event is recorded by the computer. From Rosato E. and Kyriacou C.P., 2006.

Monitoring the locomotor activity of an individual fly results in a raw data file with a number of bins (30-min recording interval) that are associated with an activity value (the number of times the fly crosses the infrared beam).

To extract the circadian period (i.e. the length of the cycle) from this file we used the chi-squared periodogram analysis. Data analysis is done on a Macintosh computer running the FaasX (Fly activity analysis suite) software, developed by M. Boudinot from the Brandeis Rhythms package.

The periodogram (**Fig.9**) is a plot of the estimation of the rhythm power as a function of the test period. The main parameters of the periodogram analysis obtained after treatment of the raw data 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 chi-squared 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.

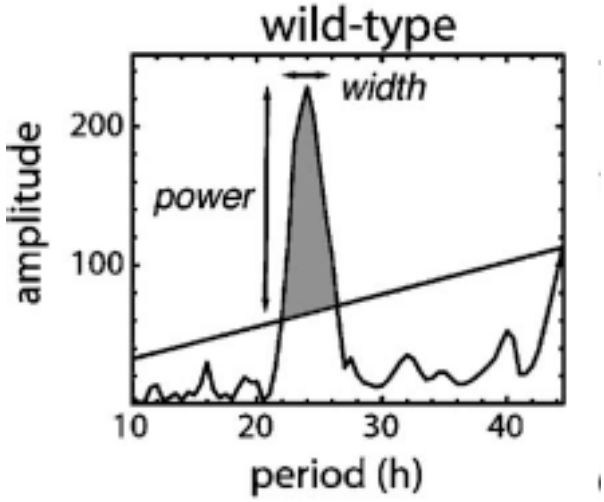


**Fig.9** Representative periodogram derived from activity records of individual flies in constant darkness. Flies were first entrained to LD cycles for 3–5 days, then kept in constant darkness for at least 6 additional days. The periodogram correspond to at least 5 days of activity, beginning 24 h after 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. From Klarsfeld A. et al., 2003.

The shown data calculated by the software FaasX were obtained using the analysis CycleP. This analysis allows the study of a single fly locomotor activity. The program calculates and shows some important rhythmic parameters such as the period of the rhythm (Tau) that is included from 18 to 30 for a rhythmic fly (a short or a long period are respectively less or more than 24 hours) and the power of the rhythm (Pwr), related to the strength of the rhythm of a single fly. A fly with a power less than 20 is considered arrhythmic. The mean of all the single powers is an index of the global population's rhythmicity. The results show also the percentage of rhythmic flies and the standard deviation of the period (sem). It is also possible to exclude, among the rhythmic flies, those that have a period too different from the average (we chose 3 hours of distance) having in this way also a percentage of rhythmic flies.

## The UAS-GAL4 system

To obtain flies with decreased expression of a particular gene, we used the UAS/GAL4 binary system. This technique is highly used to target specific spatial gene expression. The system is based on the activity of the yeast GAL4 protein to activate gene transcription from its target Upstream Activating Sequences (UAS). The expression of a gene of interest is controlled by the presence of the UAS element. Because transcription of the UAS-driven gene requires the presence of GAL4, the absence of GAL4 in the UAS lines maintains the target in a transcriptionally silent state. To activate the target gene's transcription, UAS lines are mated to flies expressing GAL4 (the driver) in a particular pattern. The resulting progeny will express the UAS-driven gene with a transcriptional pattern that reflects the driver's expression (Duffy J.B., 2002), thus according to the GAL4 promoter sequence. In our analysis, we coupled the UAS/GAL4 system with RNAi expression to target specific gene knockdown. A double inverted (palindrome) 350-500 bp sequence of the target gene is inserted downstream a UAS. The transcribed mRNA forms a hairpin that is recognized by the DICER protein and is randomly cut into several double-stranded 19-30 bp long siRNAs.

These small RNAs are then identified by the RISK protein complex that splits them into ssRNAs, which detect and pair with the endogenous mRNA of the target gene. The RISC machinery then acts again in the paired RNAs, leading to the disruption of the endogenous mRNAs and the subsequent decrease of the protein's production (**Fig.10**).

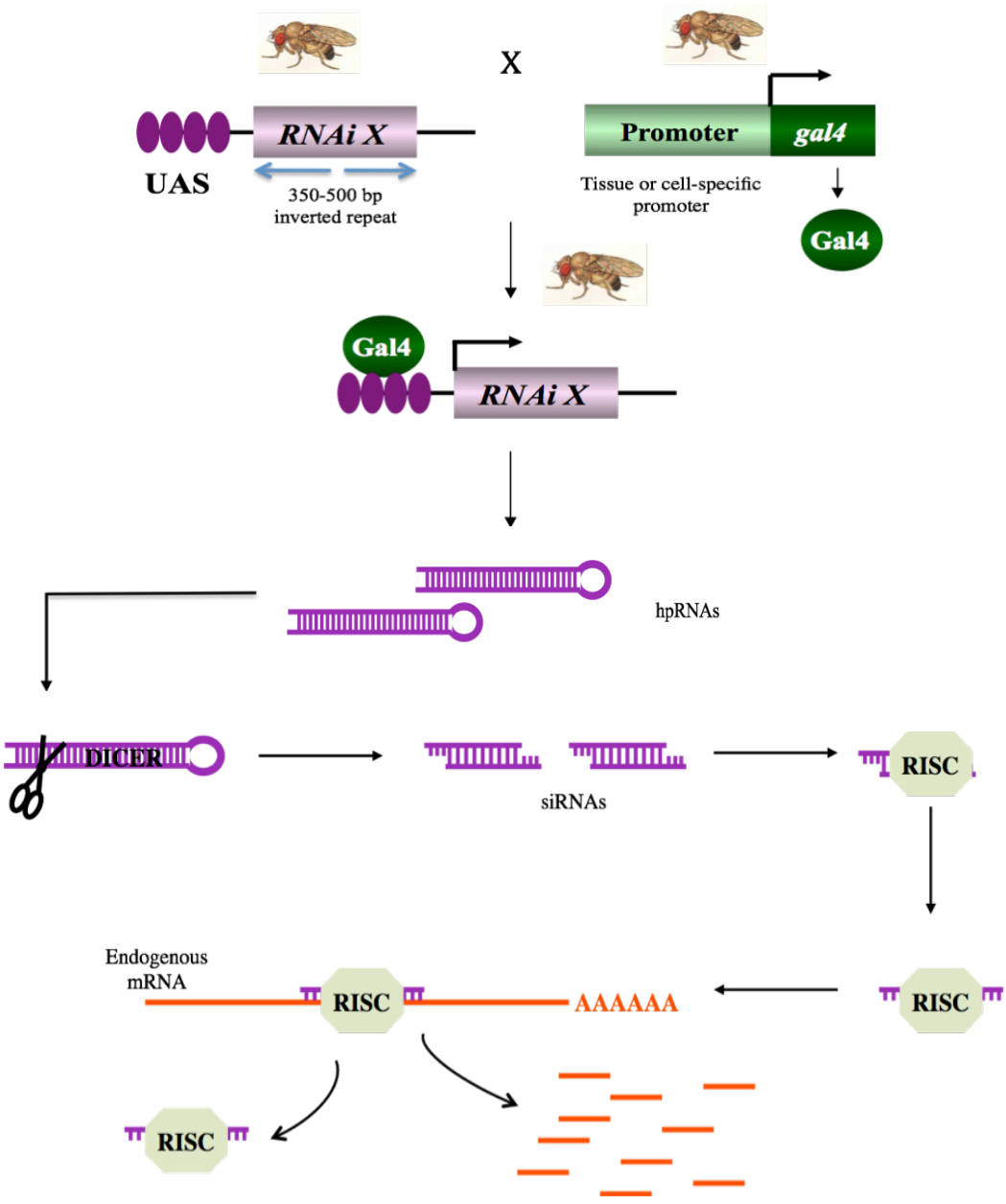


**Fig.10** The GAL4/UASRNAi system. The generic GAL4/UAS system is used to drive the expression of a hairpin RNA (hpRNAs). These double-stranded RNAs are processed by DICER into siRNAs which direct sequence-specific degradation of the target mRNA.

RNAi phenotypes sometimes corresponded to the null phenotypes reported for classical mutations, but more often resembled a hypomorphic phenotype. It has been proved that over-expression of dicer2, a protein which takes part in the interference machinery, consistently enhanced the RNA interference (Dietzl G. et al., 2007). Thus, co-expression of a *UAS-dicer2* transgene turns out to be a useful approach to enhance transgenic RNAi effects.

## CRY partners' RNAi

Using the promoter sequence of a widely expressed gene means that the RNAi machinery will be activated in many cells, increasing the possibility of induced lethality. Using a weak promoter the RNAi production could not be sufficient to mimick the correct knockdown of the target gene.

To select the proper driver promoter for our CRY partners' RNAi experience we set up a test using the GAL4 gene under two different promoters, the CRY – *crygal4-39* line, and the TIM one – *timgal4-62* line. Giving the better rhythmicity obtained when expressing a *UAS-cryRNAi* control gene, we finally decide to use the *timgal4* as driver. We used flies having an *ls-tim* genetic background, as this allele it is known to give a better rhythmicity in constant-light condition than the *s-tim* allele (Peschel N. et al., 2006). This choice lays the possibility of generate flies carrying the *Veela* ($jet^{c}$, $tim^{ls}$) genotype but the probability is not so high to invalidate the test. The *timgal4-62* driver line was provided by Jeff Hall (Brandeis University) while the *UASRNAi* reporter lines were ordered from the NIG-FLY (Japan) and the VDRC (Austria) stock centers. We used positive (*UAS-cryRNAi*, and *w;;cryb*) and negative (driver *timgal4* construct without *UASgene* construct) control lines to validate the experiments. Negative control lines were used with the same genetic background present in RNAi strains, i.e. using the wild-type strain present in the stock-centre: *wRyu* for the NIG/FLY center and *w1118* for the VDRC one. The parental lines were stored in LD 12:12 cycles at 19°C, while the crosses were made and kept at 25°C, due to the temperature dependence of GAL4 activity in *Drosophila*, which is minimal at 16°C and maximal at 29°C (Duffy J.B., 2002).

## *Drosophila* whole-genome RNAi screening

Standard conditions for the *Drosophila* whole-genome RNAi screening (NIG-FLY RNAi stock center collection) are as follows.

*UAS-RNAi* transgenic flies are mated to a *gal1118* line that drives RNAi expression in the $LN_vs$, the brain clock neurons known to be directly involved in the fly's rhythmic locomotor behaviour in constant darkness. Progeny flies are entrained for 2 days in $12^hL:12^hD$ cycles and then tested in constant darkness condition for one week at 23C. Four flies are tested for each transgenic genotype. *UAS-RNAi* flies with no GAL4 driver are taken as control to test the absence of circadian effects driven by the insertion itself. Positive genotypes, showing a period difference of more than one hour with the controls or arrhythmicity in more than 2/4 individual flies, are tested again in the same conditions. Lines with a reproducible phenotype are selected and retested with more individuals (usually 16 flies) to produce robust data. House-keeping genes are discarded and still positive lines are then outcrossed to remove double insertions (rather frequent in the RNAi collection). Outcrossed lines are behaviourally re-tested and checked for their brain clock neurons morphology by brain dissection and anti-PDF immunocytofluorecence. This eliminates genotypes which positive phenotype is just a consequence of damaged clock neurons.

## Driver lines used

**Table3** indicates the genotypic composition of the driver lines used.

The *gal4* coding gene preceded by the specific promoter is inserted in *Drosophila*'s genome as a P-element. Moreover, the insertion construct possesses a *mini-white* ($[w^+]$) gene, which give red color to the fly eyes: this permits to distinguish transgenic flies from laboratory wild-type *w* strains.

A specific *timgal4* driver line is used for the test in LL conditions. This strain has been selected in its endogenous *tim* gene allelic composition to be homozygous for the *ls-tim* allele.

| Strain | Genotype | Pattern of expression |
|---|---|---|
| *gal1118* | $\frac{w}{w};\frac{+}{+};\frac{P[w^+,??:GAL4]}{P[w^+,??:GAL4]}$ | s-$LN_v$s (not the 5$^{th}$), l-$LN_v$s; partial on $LN_d$ and DN1 |
| *timgal4* | $\frac{w}{w};\frac{P[w^+,tim:GAL4]}{P[w^+,tim:GAL4]};\frac{+}{+}$ | All clock neurons |

**Table3** Genotype denotation and pattern of expression of the driver :*gal4* lines utilized for experiments. The promoter region that drives *gal4* expression in the *gal1118*-gal4 driver has not yet been identified. See main text for details.

## Fly heads protein extraction

For each circadian time tested, almost 40-50 entrained flies were collected in different tubes and frozen at -80°C. Fly heads were removed mechanically and separated from the bodies using a suitable strainer. Protein extraction is performed using mechanic heads crushing with 4uL of extraction buffer per head.

Extraction Buffer (doses for 5mL)

| | |
|---|---|
| Paul Hardin buffer | 5 mL |
| Antiprotease | 1 tablet |
| Phosphatase inhibitor cocktail 1 | 25 uL |
| Posphatase inhibitor cocktail 2 | 50 uL |
| Beta GlyceroPhosphate 1M | 100 uL |

Paul Hardin buffer stocking solution (final concentration):

| | |
|---|---|
| Hepes | 10 mM |
| TrisHCl | 5 mM |
| KCl | 50 mM |
| Glycerol 100% | 10% |
| EDTA | 2 mM |
| Triton X-100 | 1% |
| NP40 | 0,4% |

Eppendorfs are centrifugated 15’ at 14000 rpm (max) at 4°C and the supernatant is stored at -80°C.

## Protein dosage

Protein extracts quantification is done using Bradford BioRad protein assay.

Each test is done using 1 mL of solution:

| | |
|---|---|
| $H_2O$ milliQ | 800 uL |
| BioRad5X | 200 uL |
| Sample | 2 uL |

Quantification was performed with spectrophotometer using a standard curve made with 6 samples of known IgG at growing concentration. The tests were displayed twice for each sample and the average value was taken.

## Western Blot

Electrophoresis

Migration performed with NuPAGE Nove Tris Acetate gel by Invitrogen (Acrylammide gradient 3-8%).

Composition of loaded solutions (total volume 16 or 20 uL, depending on protein samples’ concentrations):

| | |
|---|---|
| Protein samples | volume for 50 mg of protein |
| DTT 1M 10X | 1,6 – 2 mL |
| Blue NuPAGE 4X | 4 – 5 mL |
| $H_2O$ milliQ | up to final volume |

Running buffer composition (700 mL for each cuve):

| | |
|---|---|
| Invitrogen NuPAGE Tris-Acetate SDS RB 20X | 35 mL |
| Deionized $H_2O$ | 665 mL |

200 mL of Invitrogen NuPAGE antioxidant were added to this buffer for filling the upper chamber (in contact with the gel).

Migration performed at 150V constant for 3 hours to detect PER, TIM and CLK proteins and for 1,5 hours to detect VRI and PDP1e proteins.

## Transfer

Transfer buffer composition (dosage for 700 mL – two boxes):

| | |
|---|---|
| Invitrogen NuPAGE Transfer Buffer 20X | 35 mL |
| Methanol 100% | 70 mL |
| Invitrogen NuPAGE Antioxidant | 700 mL |
| Deionized $H_2O$ | up to final volume. |

We use Invitrogen PVDF transfer membranes. Each membrane was briefly washed in methanol (10 sec), then in $H_2O$. Blotting pads, PVDF transfer membrane and filter paper were tempered in Transfer buffer before deposition. The upper chamber was filled with Transfer buffer, whether the lower chamber was filled with deionized $H_2O$. Transfer was performed for 1 hour at 30V constant. Correct transfer was assured by analysis using Ponceau red.

## Incubation with antibodies

Buffer TBS Tween 0,3% 1X composed by:

| | |
|---|---|
| NaCl | 16,4 g |
| Tris Hcl 0,5M pH 7,5 | 20 mL |
| Tween 20 | 1 mL |
| Deionized $H_2O$ | up to 2 L of final volume. |

Milk solution for incubation with antibodies was obtained by adding 5% powered milk to TBST buffer.

After a brief wash on TBST buffer, membranes were pre-incubated for at least 1 hour at RT. Then incubation with primary antibodies in milk solution was performed as follow:

| **Primary Ab** | **Origin** | **Primary Ab Conc.** | **Incubation with Primary Ab** | **Secondary Ab Conc.** |
|---|---|---|---|---|
| aPER | Rabbit | 1/10000 | 1 to 3 night(s), 4°C | 1/10000 |
| aTIM | Rat | 1/2000 | 1 night, 4°C | 1/20000 |
| aCLK | Goat | 1/1000 | 1 to 3 night(s), 4°C | 1/20000 |
| aPDP1e | Guinea Pig | 1/5000 | 1 night, 4°C | 1/10000 |
| aVRI | Guinea Pig | 1/5000 | 1 night, 4°C | 1/10000 |

After washing 5 times for 5-10 min in TBST, membranes were incubated with secondary antibody conjugated to peroxidase for 1 hour at RT. After that, 5 washes with TBST were repeated.

Colorimetric reaction was performed using GE Healthcare ECL+ WBlotting detection system, 5 minutes on the membranes. The development of the reaction was revealed using Kodak GBX developer/replenisher (1X final concentration) and GE Healthcare Amersham Hyperfilm ECL films at the darkroom. Fixation was performed using Kodak X-ray fixer (1X final concentration).

## Immunolabelling

### Antibodies

Primary antibodies utilized:

| Protein recognized | Origin | Concentration |
|---|---|---|
| CLK | Guinea Pig | 1/15000 |
| PDF | Mouse | 1/50000 |
| PERIOD | Rabbit | 1/15000 |
| TIM | Rat | 1/10000 |

Respectively detected with the secondary antibodies:

| Protein recognized | Couple fluorochrome | Concentration | Emission light spectrum colour |
|---|---|---|---|
| Guinea Pig IgG | Alexa 488 | 1/2000 | Green |
| Mouse IgG | Alexa 488 | 1/2000 | Green |
| Rabbit IgG | FluoProbe 546 | 1/5000 | Red |
| Rat IgG | Alexa 647 | 1/5000 | Far red |

### Buffers composition

PBS: NaCl 1,5M 100 mL
Tampon phosphate 1M pH 7,4 10 mL
Deionized water up to volume

PFA: Paraformaldehyde 4% + PBS

OX-PBS pH 7,3: NaCl 8 g
KCl 200 mg
$KH_2PO_4$ 200 mg
$Na_2HPO_4$ 5,67 g
Thimerosal 100 mg

OX-PBS T 1%: OX-PBS + Triton 1%

OX-PBS T 0,3%: OX-PBS + Triton 0,3%

BSA: Bovine Serum Albumine

Immunofluorescence in whole brains

Adult flies are plunged for few seconds in ethanol 95% and then dissected in the PBS buffer. Larvae are directly dissected in PBS buffer.
Adult as well as larval brains are then treated with the same following protocol.

| | |
|---|---|
| Fixation: | PFA 4% for 1h (adults) or 30-45 min (larvae) at room temperature (RT); |
| Washing: | OX-PBS T 0,3%, 6 times for 10 min each, RT; |
| Permeabilization: | OX-PBS T 1%, 10 min, RT; |
| Blockage: | OX-PBS T 0,3% + BSA 1%, 2h at RT or overnight at 4°C; |
| Primary Abs: | OX-PBS T 0,3% + BSA 0,1% + Antibodies, overnight or untill 72h, 4°C. |
| Washing: | OX-PBS T 0,3%, 6 times for 10 min, RT; |
| Blockage: | OX-PBS T 0,3% + BSA 1%, 1h at RT; |
| Secondary Abs: | OX-PBS T 0,3% + BSA 0,1% + Antibodies, 3h at RT or overnight at 4°C. Keep in a dark environment. |
| Washing: | OX-PBS, 6 times for 10 min at RT in a dark environment. |

Brains are then pressed between slide and cover-slide with Invitrogen ProLong Gold antifade reagent solution and sealed with nail polish. Slides are kept in darkness 24h at RT before observation and conserved at 4°C for a week or at -20°C for longer time.

Epifluorescence Microscopy and signal quantification

Fly brain fluorescence signals were analyzed with a Zeiss Imager Z1 fluorescence microscope equipped with an AxioCam MRm (Zeiss) and a module ApoTome.
In conventional fluorescence microscopy, the image always consists of signal contributions coming from the focused object plane and from the structures located above and below it. The blurred structures from above or below are either perceived as being out of focus or, if they are clearly outside the focal plane, have the effect of brightening the image background, which reduces image contrast. In the "structured illumination" process used in ApoTome (Carl Zeiss), a 3-position lateral moving grid is inserted into the field diaphragm plane of the fluorescence beam path (**Fig.11**). Superimposing the projected grid onto the fluorescent sample generates an image of the specimen that has dark grid lines running through it (**Fig.12**). Outside the focal plane the grid is also blurred, thus no grid projection is visible in defocused specimen structures (**Fig.12A**, arrow). In the context of image analysis, this provides a criterion for differentiating in-focus object structures from those that are defocused. The determination of the grid contrast as a function of the location (xy) can thus be used to calculate an optical section (**Fig.12D**). In order to reconstruct all the image information, at least three raw images with different positions of the grid projections must be acquired (**Figs.12A-C**: grid shift marked by blue line). (Bauch H. et al., 2006).

Fluorescence intensity was quantified from digital images with the ImageJ software. To count for the different exposition times (and thus background intensity) used, we applied the formula: I= 100 x (S-B) / B, that gives the fluorescence percentage above background (S: fluorescence intensity, B: average intensity of the region adjacent to the positive cell).

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**Fig.11** Structured illumination with the ApoTome. The fluorescence excitation light passes through two glass plates in the ApoTome slider. There is a grid structure on the first glass plate; the grid pattern is "imprinted" in the excitation light. The second (plane-parallel) glass plate is tilted by a scanning mechanism, thereby slightly shifting the beam path with the imprinted grid pattern. The excitation light (green) with the grid pattern is then projected onto the specimen via a conventional fluorescence filter set. The emission light (red) is collected by the objective and imaged onto the camera sensor. From Bauch H. et al., 2006.

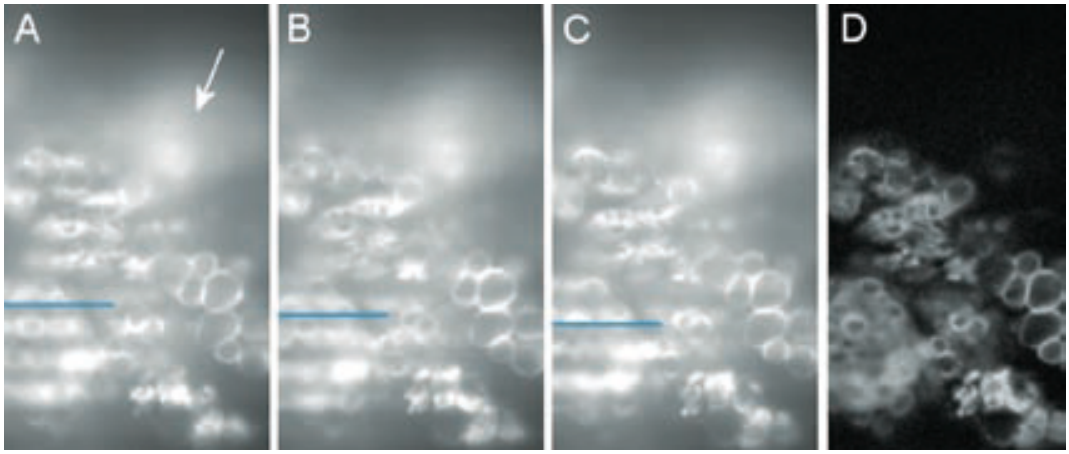



**Fig.12** In structured illumination, three row images of the fluorescent specimen are acquired, each with a different grid position (**A-C**). An image-analysis algorithm calculates an online optical section (**D**) through the specimen from the row images. See main text. From Bauch H. et al., 2006

# RESULTS

## Behavioural analysis of putative CRY partners

**Table4** and **Table5** show all the RNAi lines analyzed and the locomotor activity data obtained from the corresponding transgenic flies. Behavioral activity is expressed in terms of rhythmicity and period length (see **Table4** *caption* for details).

The experiment is aimed at finding genotypes in which the candidate gene RNAi-induced downregulation mimics a CRY mutant, that is rhythmicity in constant-light conditions. As can be seen from the tables, the tested genes show no or little rhythmicity in LL conditions. Even in the case of high rhythmicity percentage, the high standard deviation (> 0,5) means that each fly behave with a different locomotor rhythm and the rhythmicity displayed could not be taken as significant for the genotype ($per^0$ flies display a similar phenotype in DD condition for example). These flies are thus considered arrhythmic. Positive (*w/Y;timgal4/3772R-3* that drives cry-RNAi, and *w/Y;;cryb* mutant) and negative (*wRyu/Y;timgal4/+* and *w1118/Y;timgal4/+*, respectively, with the NIG/FLY and the VDRC background genotype) control flies behaved as expected, validating the correct setup of the experiment. It is worth to remember that the production of double-stranded mRNA usually does not completely suppress gene expression. This means that we do not expect to see a mutant phenotype with genes for which a very low amount of protein is sufficient to maintain wild-type activity. One way to increase the production of double-stranded mRNA, hence the down-regulation of the target gene, is to produce homozygous lines either for the RNAi or both the driver and the RNAi insertions. This requires several months of work, mostly to generate recombinant strains carrying both the *gal4* and the *UAS-RNAi* transgenes. Dicer2 over-expression have been linked to an increased RNAi

induction, thus a more reduced endogenous target-protein production. We thus decided to combine RNAi + dicer2 expression to enhance the RNAi effects. A driver line carrying both the *timgal4* and the *UAS-dicer2* transgene has been constructed. This strain will be crossed with *UAS-RNAi* expressing flies and the progeny will be tested as previously (LL conditions, 23°C) (work in progress).

| Gene | Genotype tested | Alive flies (tested) | % R | Tau (Sem) | Power (Sem) |
|---|---|---|---|---|---|
| **RNAi** | | | | | |
| CG7998 | w/Y;*timgal4*/+;22654/+ | 16 (16) | 43,8 % | 26,0 (1,55) | 36,3 (6,80) |
| | 7998/Y; *timgal4*/+ | 16 (16) | 68,8 % | 29,3 (1,75) | 49,5 (7,71) |
| CG8824 | w/Y;*timgal4*/+;4638/+ | 16 (16) | 62,5 % | 32,3 (2,43) | 30,4 (4,08) |
| | w/Y;*timgal4*/+;4637/+ | 16 (16) | 81,3 % | 24,5 (2,12) | 46,7 (5,19) |
| CG3874 | w/Y;*timgal4*/+;47543/+ | 15 (16) | 53,3 % | 29,3 (1,14) | 59,2 (9,61) |
| | w/Y;*timgal4*/47542 | 9 (9) | 66,7 % | 29,3 (0,75) | 44,8 (8,13) |
| CG2224 | w/Y;*timgal4*/20852 | 16 (16) | 68,8 % | 25,3 (2,02) | 43,7 (5,86) |
| CG32031 | w/Y;*timgal4*/+;34036/+ | 16 (16) | 56,3 % | 23,3 (1,52) | 55,8 (10,25) |
| | w/Y;*timgal4*/+;34037/+ | 16 (16) | 50,0 % | 30,3 (1,61) | 47,2 (9,01) |
| CG4757 | w/Y;*timgal4*/38051 | 16 (16) | 31,3 % | 25,3 (2,45) | 53,5 (10,66) |
| | w/Y;*timgal4*/38052 | 16 (16) | 81,3 % | 28,4 (1,34) | 50,7 (6,43) |
| CG1905 | w/Y;*timgal4*/+;48769/+ | 16 (16) | 12,5 % | 27,8 (0,25) | 56,6 (22,28) |
| | w/Y;*timgal4*/48768 | 16 (16) | 50,0 % | 27,8 (1,63) | 45,3 (8,92) |
| CG1066 | w/Y;*timgal4*/+;46889/+ | 16 (16) | 62,5 % | 27,9 (2,99) | 34,6 (3,65) |
| | w/Y;*timgal4*/46887 | 13 (16) | 38,5 % | 27,4 (1,39) | 38,9 (4,75) |
| CG30437 | w/Y;*timgal4*/+;22959/+ | 16 (16) | 43,8 % | 26,8 (1,47) | 51,7 (13,47) |
| CG4000 | w/Y;*timgal4*/+;16671/+ | 16 (16) | 75,0 % | 28,3 (0,71) | 47,5 (6,14) |
| CG3805 | w/Y;*timgal4*/+;26337/+ | 16 (16) | 62,5 % | 29,5 (1,04) | 40,9 (5,08) |
| | w/Y;*timgal4*/+;26336/+ | 16 (16) | 43,8 % | 26,7 (1,39) | 61,9 (11,38) |
| CG31628 | w/Y;*timgal4*/+;46295/+ | 16 (16) | 87,5 % | 22,6 (1,85) | 42,2 (4,23) |
| | w/Y;*timgal4*/+;46293/+ | 15 (16) | 73,3 % | 25,6 (2,44) | 42,7 (4,82) |
| CG10106 | w/Y;*timgal4*/7934 | 16 (16) | 56,3 % | 27,2 (2,40) | 36,5 (3,96) |
| CG10966 | w/Y;*timgal4*/20100 | 16 (16) | 62,5 % | 28,3 (2,79) | 39,4 (6,72) |
| **controls** | | | | | |
| cry-RNAi | w/Y;*timgal4*/3772R-3 | 16 (16) | 100 % | 23,4 (0,15) | 133,8 (9,88) |
| driver | w1118/Y;*timgal4*/+ | 15 (17) | 46,7 % | 27,4 (1,94) | 53,3 (8,43) |
| $cry^b$ | w/Y;;cryb | 13 (16) | 76,9 % | 23,7 (0,29) | 77,0 (9,56) |

**Table4** Locomotor activity analysis of transgenic flies (*UAS-RNAi* lines from the VDRC stock centre). The table show the genotype(s) tested for each target gene and the number of flies that reach the end of the experiment (on brackets, the number of flies initially put on the activity monitor). The percentage of rhythmic behavior is referred to the number of alive flies. Tau expresses period's length. The power column describe the robustness of the period's length; in general, periods with a standard deviation (Sem) more than 0,8 are regard as an absence of rhythmicity because that means flies with the same genotype behave extremely differently, which could not be considered as a significant rhythm.

**Table5** Locomotor activity analysis of transgenic flies (*UASRNAi* lines from the NIG-FLY stock centre) (see Table4 captation for details).

| Gene | Genotype tested | Alive flies (tested) | % R | Tau (Sem) | Power (Sem) |
|---|---|---|---|---|---|
| **RNAi** | | | | | |
| CG31149 | w/Y;*timgal4*/5449R-2 | 13 (16) | 23,1 % | 31,5 (5,03) | 31,3 (3,16) |
| | w/Y;*timgal4*/5449R-1 | 6 (7) | 16,7 % | 30,0 (0,00) | 65,0 (0,00) |
| CG4696 | w/Y;*timgal4*/+;4696R-1/+ | 14 (16) | 28,6 % | 20,8 (3,15) | 37,0 (6,91) |
| | w/Y;*timgal4*/4696R-4 | 12 (16) | 33,3 % | 28,5 (1,50) | 42,6 (7,84) |
| CG5390 | w/Y;*timgal4*/5390R-1 | 16 (16) | 56,3 % | 23,8 (2,54) | 36,9 (4,49) |
| CG31795 | w/Y;*timgal4*/11344R-4 | 16 (16) | 75,0 % | 30,8 (1,52) | 46,1 (6,45) |
| | w/Y;*timgal4*/+;11344R-3/+ | 8 (8) | 37,5 % | 21,3 (4,06) | 46,0 (14,65) |
| | w/Y;*timgal4*/+;11344R-3*/+ | 8 (8) | 87,5 % | 23,6 (2,48) | 46,1 (6,31) |
| CG4550 | w/Y;*timgal4*/+;4550R-2/+ | 11 (12) | 90,9 % | 20,5 (1,88) | 40,3 (6,68) |
| | w/Y;*timgal4*/4550R-1 | 7 (7) | 57,1 % | 21,3 (2,33) | 36,6 (5,85) |
| | w/Y;*timgal4*/4550R-1;4550R-1?/+ | 16 (16) | 43,8 % | 23,1 (1,85) | 33,5 (3,65) |
| CG7470 | w/Y;*timgal4*/7470R-3 | 16 (16) | 62,5 % | 29,5 (1,73) | 47,3 (8,69) |
| CG4662 | w/Y;*timgal4*/+;4662R-3/+ | 11 (13) | 27,3 % | 28,0 (0,29) | 44,4 (10,79) |
| | w/Y;*timgal4*/4662R-4 | 15 (16) | 46,7 % | 30,9 (0,97) | 55,2 (7,99) |
| CG2985 | w/Y;*timgal4*/2985R-1 | 16 (16) | 12,5 % | 28,5 (3,50) | 37,7 (11,10) |
| | w/Y;*timgal4*/+;2985R-2/+ | 15 (16) | 73,3 % | 21,9 (2,08) | 41,9 (4,22) |
| CG5939 | w/Y;*timgal4*/5939R-2 | 16 (16) | 37,5 % | 28,8 (1,85) | 40,2 (8,23) |
| | w/Y;*timgal4*/5939R-3 | 16 (16) | 37,5 % | 21,0 (2,67) | 31,2 (4,85) |
| CG8663 | w/Y;*timgal4*/8663R-1 | 16 (16) | 31,3 % | 23,6 (3,44) | 43,4 (4,45) |
| | w/Y;*timgal4*/+;8663R-2/+ | 16 (16) | 12,5 % | 32,0 (4,50) | 42,7 (16,47) |
| CG4264 | w/Y;*timgal4*/4264R-1 | 0 (8) | - | - | - |
| CG17725 | w/Y;*timgal4*/+;17725R-1/+ | 16 (16) | 43,8 % | 21,7 (2,73) | 38,0 (8,34) |
| | w/Y;*timgal4*/17725R-2 | 14 (16) | 35,7 % | 23,9 (4,56) | 36,8 (8,32) |
| CG12317 | w/Y;*timgal4*/12317R-1 | 11 (11) | 72,7 % | 23,4 (2,85) | 43,7 (3,53) |
| | w/Y;*timgal4*/12317R-4 | 15 (16) | 66,7 % | 23,2 (2,45) | 40,1 (4,09) |
| CG10067 | w/Y;*timgal4*/+;10067R-2/+ | 16 (16) | 43,8 % | 25,4 (2,00) | 51,7 (8,55) |
| | w/Y;*timgal4*/+;10067R-1/+ | 13 (16) | 18,8% | 30,5 (4,33) | 37,0 (8,34) |
| CG8804 | w/Y;*timgal4*/8804R-1 | 14 (16) | 28,6 % | 27,8 (5,14) | 33,7 (4,58) |
| | w/Y;*timgal4*/+;8804R-3/+ | 15 (16) | 46,7 % | 26,9 (2,46) | 37,5 (6,80) |
| CG10596 | w/Y;*timgal4*/+;10596R-1/+ | 14 (16) | 21,4 % | 29,0 (4,04) | 40,9 (8,94) |
| | w/Y;*timgal4*/10596R-2 | 16 (16) | 43,8 % | 22,8 (1,55) | 31,5 (2,75) |
| CG11909 | w/Y;*timgal4*/+;11909R-1/+ | 16 (16) | 43,8 % | 27,9 (4,48) | 41,2 (3,75) |
| | w/Y;*timgal4*/11909R-3 | 15 (16) | 46,7 % | 27,1 (4,21) | 32,1 (3,71) |
| CG31689 | w/Y;*timgal4*/31689R-1 | 16 (16) | 43,8 % | 23,4 (3,78) | 41,5 (5,96) |
| | w/Y;*timgal4*/+;31689R-2/+ | 15 (16) | 46,7 % | 27,9 (3,29) | 43,9 (3,32) |
| CG6151 | w/Y;*timgal4*/+;6151R-2/+ | 16 (16) | 50,0 % | 23,8 (3,52) | 41,9 (3,71) |
| | w/Y;*timgal4*/6151R-4 | 16 (16) | 56,3 % | 28,9 (3,33) | 42,8 (6,82) |
| CG8588 | w/Y;*timgal4*/8588R-1 | 16 (16) | 81,3 % | 26,2 (1,98) | 45,7 (5,63) |
| | w/Y;*timgal4*/+;8588R-4/+ | 16 (16) | 68,8 % | 23,0 (2,01) | 43,6 (5,60) |
| CG6806 | w/Y;*timgal4*/+;6806R-2/+ | 16 (16) | 50,0 % | 21,8 (3,69) | 62,9 (14,35) |
| | w/Y;*timgal4*/6806R-3 | 15 (16) | 40,0 % | 20,2 (1,26) | 46,4 (9,75) |
| CG34418 | w/Y;*timgal4*/5406R-2 | 16 (16) | 43,8 % | 26,6 (2,38) | 40,9 (5,65) |
| | w/Y;*timgal4*/+;5406R-3/+ | 13 (14) | 38,5 % | 24,5 (1,05) | 43,4 (5,61) |
| CG2259 | w/Y;*timgal4*/+;2259R-1/+ | 11 (12) | 63,6 % | 26,8 (3,34) | 40,3 (5,71) |
| | w/Y;*timgal4*/+;2259R-3/+ | 15 (16) | 6,7 % | 16,5 (0,00) | 27,5 (0,00) |
| CG16935 | w/Y;*timgal4*/+;16935R-1/+ | 14 (16) | 92,9 % | 25,3 (1,73) | 53,9 (6,92) |
| | w/Y;*timgal4*/16935R-2 | 14 (15) | 64,3 % | 22,6 (2,09) | 42,5 (10,34) |
| CG5125 | w/Y;*timgal4*/5125R-2 | 15 (16) | 66,7 % | 25,5 (2,89) | 37,1 (5,06) |
| **Controls** | | | | | |
| cry-RNAi | w/Y;*timgal4*/3772R-3 | 16 (16) | 100 % | 23,4 (0,15) | 133,8 (9,88) |
| driver | wRyu/Y;*timgal4*/+ | 16 (16) | 75,0 % | 28,1 (1,99) | 49,5 (4,58) |
| $cry^b$ | w/Y;;cryb | 13 (16) | 76,9 % | 23,7 (0,29) | 77,0 (9,56) |

## USP5

*Usp5* lacking flies display a longer period when free-running in constant darkness. In these conditions, protein quantification analysis have demonstrated that the flies show an aberrant persistence of PER, TIM and CLK proteins when their levels are normally minimal, meaning in the middle/end of the day for PER and TIM, and at the end of the day/beginning of the night for CLK. This indicates either an abnormal accumulation or a deficient degradation. To understand whether Usp5 would only affect light-independent degradation, we asked whether this phenomenon is still observable when the light-entraining stimulus is present.

How do flies expressing *usp5*-RNAi behave in the presence of the light-entraining stimulus?

- Locomotor activity in standard 12:12 h LD conditions

To ask whether Usp5 plays a role in locomotor activity rhythms in LD conditions, *usp5*-RNAi double-inserted flies were kept in 12:12 h LD conditions for 10 days and tested for the presence of morning and evening anticipation peaks (**Fig.13**). The production of the RNAi was driven by two different drivers: *timgal4*, which is expresses in all clock neurons, and *gal1118*, whose expression is mostly restricted to the s- and l-$LN_v$s (plus a weak expression in 3 $LN_d$ and a few DNs).
In LD conditions, light is able to reset *period* mutant flies, which display an abnormal (longer or shorter) period in constant darkness conditions, to the 24-hours environmental cycling. However, differently from wild-type flies, the evening anticipatory activity of these flies is phase-shifted according to the alteration of the period in DD (Hamblen-Coyle M.J. et al., 1992). When analyzed for LD activity, long-period (tau= 29h) $per^L$ mutants display an evening activity peak that is delayed from the end of the day to the beginning of the night. Moreover, they frequently lack the morning peak, which is supposed to be masked by the activity induced by lights-on. Similarly, we expected to see a delayed

anticipatory activity in the *usp5-RNAi* flies. On contrary, our transgenic flies behave very similarly to controls, showing a normal increase of locomotor activity before light transitions both with the *timgal4* and the *gal1118* drivers. We can imagine that clock proteins mutants (such as $per^L$) possess a stronger phenotype as they only have the mutant protein and they have it in all cells. Transgenic RNAi flies, on contrary, just decrease protein levels and, moreover, do it only in the cells that express the driver.

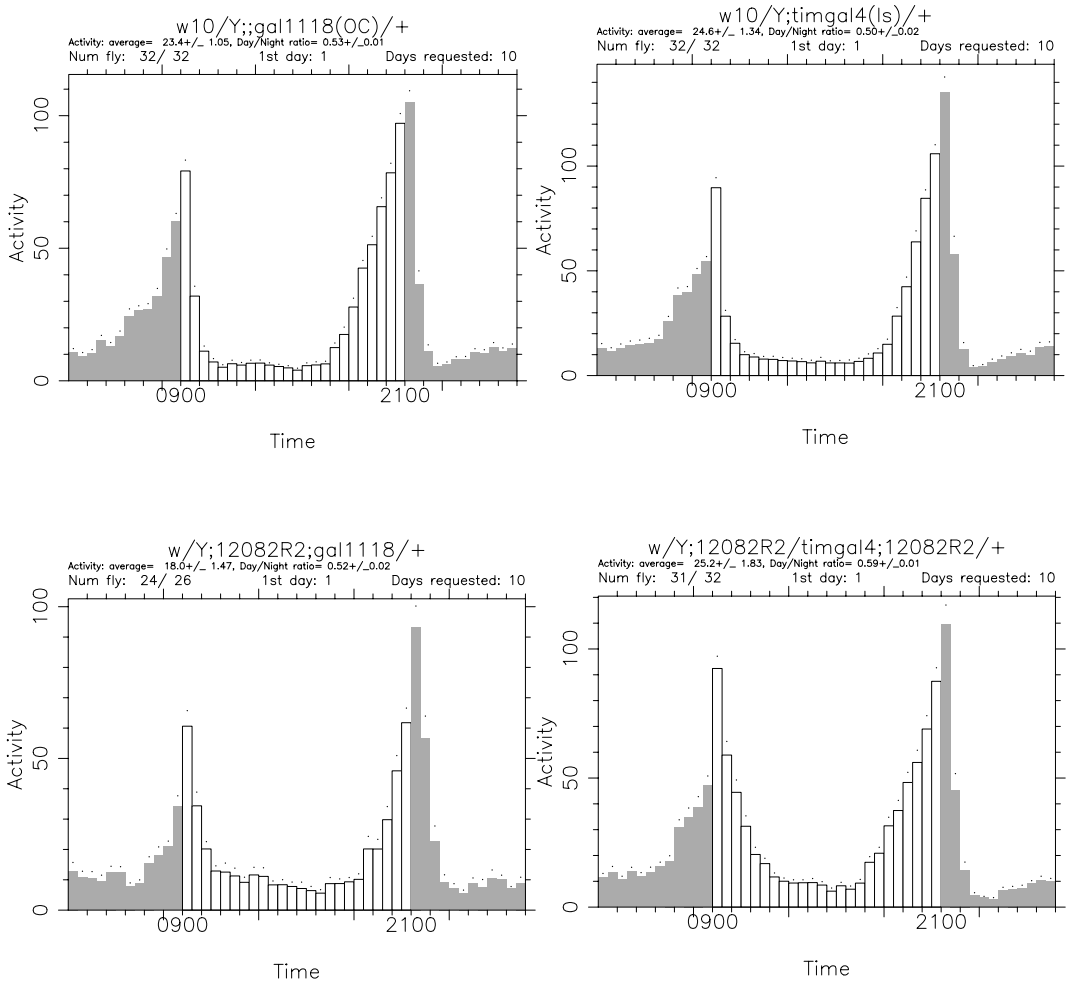



**Fig.13** Eduction (24-hours plot of the average locomotor activity measured every 30 min of each day) derived from a group of flies taken for 10 days in LD conditions. For each graphic it is shown the genotype and the number of flies tested. Graphics should be compared vertical: control (carrying only the driver insertion) flies on the top, Usp5-RNAi flies on the bottom. On the right, *timgal4* driver; on the left *gal1118* driver. X-axis: time; Y-axis: average of activity.

In general, proper morning cells (M-cells) activity sustains the master clock ticking during constant darkness condition and is responsible of setting the time for morning events to occur. On contrary, evening cells (E-cells) are required to set the time during constant light conditions and control evening activity outputs. In standard LD conditions, morning and evening oscillators thus have to cooperate to time day and night events, respectively, with a switch between morning and evening cell predominance (Picot M., 2007; Stoleru D. et al., 2007). Under 12:12 h LD conditions, speeding up specifically the E-cells clock, by targeted over-expression of SGG, advanced not only the evening peak but also the morning anticipation. Instead, fast M-cells have no significant effects on the phase of evening outputs. E-cells thus possess a certain predominance in controlling evening and also morning outputs in 12:12 h light:dark cycles (Stoleru D. et al., 2007). Similarly to a fast one, we can suppose that a slow E-cells clock induces a delay in evening and morning peaks. The *gal1118* driver drives RNAi expression specifically in the M-cells (s- and l-$LN_vs$), which could explain why, according to this hypothesis, we don't see an altered phenotype. The *timgal4* driver, instead, drives RNAi expression in all clock cells, including the evening oscillator cells. Unfortunately, when tested in total darkness conditions these flies manifest only 1.5 hours of delaying (tau=25h), which is very presumably due to a weaker RNAi expression (compared to *gal1118*). This mild phenotype can thus not render visible *usp5*-RNAi effects on the morning and evening peak of activity. An alternative hypothesis is that light controls the activation of other mechanisms that act to compensate Usp5 lack of function. These light-activated mechanisms would thus mask the effects of Usp5 absence during light:dark cycles. Generating a *timgal4* phenotype with a stronger period change would allow concluding about Usp5 function in LD.

- Locomotor (ar)rhythmicity in LL conditions

RNAi-expressing flies have been even tested for arrhythmicity in constant light conditions (**Table6**). To increase the possibility of detecting poor rhythmic phenotypes we used the *timgal4* driver in an *ls-tim* genetic background. (see *Materials and Methods*).
In these conditions, Usp5-RNAi flies are extremely arrhythmic, as for controls. This strongly suggests that *usp5* down-regulation does not prevent TIM degradation by light.

| **Genotype** | **Alive flies (tested)** | **% R** | **Tau (sem)** | **Power (sem)** |
|---|---|---|---|---|
| w;12082$^{NIG}$/ *timgal4*;12082$^{NIG}$/+ | 14 (15) | 0,00% | - | - |
| w;*timgal4*/+ | 16 (16) | 18,80% | 26,7 (6,21) | 25,5 (2,89) |

**Table6** Locomotor behaviour analysis of *usp5*-RNAi flies taken in constant light conditions (LL) for 10 days after 2 days of entraining LD 12:12 h. The data refers to the analysis from LL3 to LL10. 'Alive flies' means those that survive until the end of the experiments among the flies put initially on monitors (tested). Rhythmicity percentage is calculated among the survived flies. Tau expresses the period's length and the power indicates the robustness of the rhythmicity displayed.

## Do clock proteins in RNAi flies still cycle as expected?

In order to analyze the effect of Usp5 on *Drosophila* molecular clock we used two different approaches: immunostaining of clock proteins in dissected adult fly brains and western blot analysis of total fly head protein extracts.

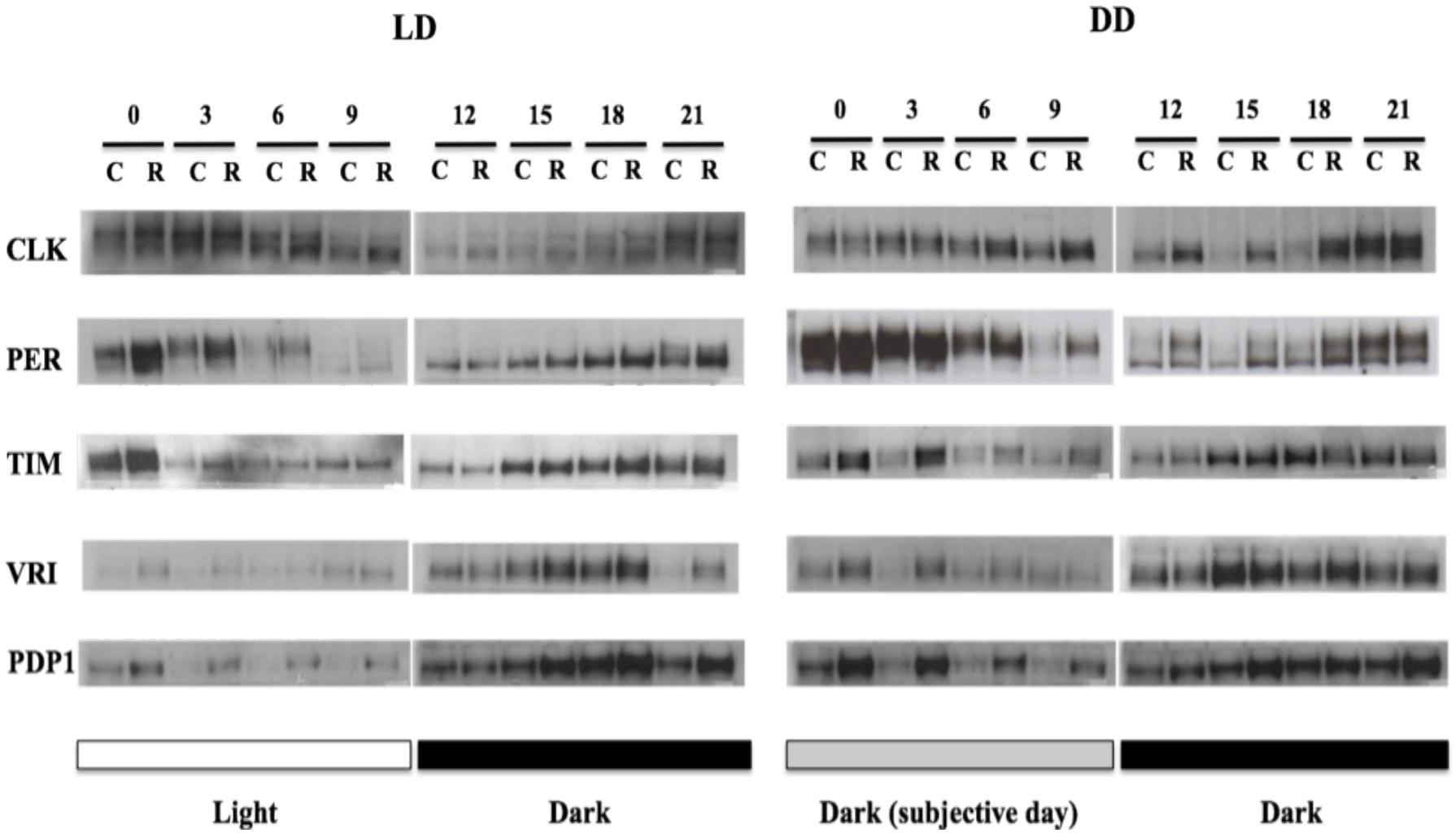
- Western Blots analysis

When total fly head extracts are analyzed, RNAi expression in cells must be driven more ubiquitously, as clock neurons represent only a small percentage of the head cells expressing clock proteins, whose most important contribution is

provided by the eye photoreceptors. This is why for these experiments *usp5-RNAi* induction is driven by *timgal4*, which is expressed in most clock cells, including brain clock neurons and peripheral clock cells. Western blot analysis of fly head extracts representing the first day of DD have already shown an altered PER and TIM protein persistence during the day. To ask whether it is still the case when the light-resetting stimulus is present, we analyzed fly head extracts from the last day of LD entrainment, whereas the first day of DD was used as an internal control (**Fig.14**). Head extracts were prepared from flies collected every 3 hours throughout the last day of LD and the first day of darkness. The same experiment protocol has been performed twice. We obtained comparable results, thus attesting a reproducible effect of Usp5.

During DD1, we observe a general persistence of clock proteins in flies expressing the *usp5*-RNAi. In particular, PER and TIM proteins accumulate during the night but fail to be normally degraded during the pseudo-day. We noticed a persistence of higher hyper-phosphorylated PER protein isoforms at the end of the pseudo-day and at the beginning of the night. Generally, PER phosphorylation is associated to the progressive protein degradation (thought to be mediated by the proteasome after SLMB-dependent ubiquitination). As for PER and TIM, CLK protein abnormally accumulates at the end of the pseudo-day and during the night. *Usp5*-RNAi induces a stabilization of the low, hypo-phosphorylated forms of CLK. CLK phosphorylation is correlated with its inability to bind the DNA, whereas hypo-phosphorylation is generally associated with CLK being transcriptionally active. We thus quantified the proteins made from the other CLK transcriptional target genes, VRI and PDP1epsilon. As for PER and TIM, *usp5*-RNAi transgenic flies show higher levels of these proteins, which fail to be degraded as they do in control flies.

**Fig.14** Western blot analysis of the major clock proteins (CLK, PER, TIM, VRI and PDP1epsilon) during standard 12:12h LD conditions and in constant darkness (DD) conditions (shown by white-black bar or grey-black boxes, respectively, on the bottom of the figure). Head extracts from control (C) and RNAi-expressing (R) flies are put one next to the other. The numbers wrote on the top indicate the times when flies were collected during their LD or DD cycle (respectively, ZT and CT). Protein phosphorylated forms are more heavy and migrate less.


LD
DD
0 3 6 9 12 15 18 21
C R C R C R C R C R C R C R C R
CLK
PER
TIM
VRI
PDP1
Light
Dark
Dark (subjective day)
Dark

Compared to DD, flies collected during LD cycles still show an accumulation of clock proteins at the end of the night, but they undergo a stronger degradation when the light is switched-on. This is particularly true for TIM protein, which light-induced CRY-mediated degradation is apparently still active in *usp5* defective flies. PER degradation in RNAi flies during the light time appears to be less efficient compared to controls, but the same protein quantity between RNAi and control flies is finally reached at the end of the day (ZT9-12). CLK protein cycling seems to be equally perturbed as controls during LD conditions, except for a slightly less strong degradation at the end of the day. Differently from PER and TIM, VRI and PDP1epsilon are not affected by stronger protein destruction in the presence of light.

The major differences between RNAi and control flies are thus remarked when in constant darkness conditions and, in particular, for PER, TIM and CLK at those times when proteins normally reach their minimum quantities. Notably, in *usp5*-RNAi flies, clock proteins undergo the expected post-translational modifications; the only difference is shown for PER, whose hyper-phosphorylated isoforms persist, in DD conditions, during all the cycle.

Two (not exclusive) possibilities exist that can explain the abundance of clock proteins during constant darkness conditions. (1) Usp5 plays a (direct?) role in the regulation of clock proteins degradation. An impaired degradation program could thus account for CLK, PER and TIM accumulation in Usp5-depressed flies. (2) Usp5 is involved in the destruction of the hypo-phosphorylated, active forms of the CLK transcription factor, which controls *per* and *tim* (in addition to *vri* and *pdp1epsilon*) genes expression. PER and TIM proteins accumulation is thus possibly due to a higher gene transcription in *usp5*-RNAi flies. To test this hypothesis, we have started to quantify the mRNA levels of the CLK-controlled genes. Preliminary results indicate that transcription seems not to be affected.

Importantly, PER, TIM and (maybe) CLK proteins degradation is differently affected, in the presence of *usp5*-RNAi, when in DD or LD conditions. We thus suppose that Usp5 plays a role specifically during circadian-controlled protein cycling (that is, in DD conditions). This distinction implies the existence of specific degradation programs (at least for the major clock proteins) that are

differently engaged in the presence or absence of the light-stimulus. In particular, they can (at least in part) cooperate to control the correct clock protein turnover, or light can activate further degradation mechanisms to the clock-controlled ones. Accordingly to this hypothesis, a damaged "darkness" degradation protocol (caused by *usp5* down-expression) explains why clock proteins destruction is not complete during LD cycles. On contrary, VRI and PDP1epsilon protein oscillations appear to be equally perturbed in constant darkness as well as in light-dark cycles. This suggests that light does not participate to VRI and PDP1epsilon protein cycling.

- Head dissections

When directed specifically to the PDF-expressing $LN_v$s (*gal1118* driver), RNAi against endogenous *usp5* mRNA induces a slowing-down of the clock, which is set to a period of about 28-29 hours. This lengthened period in locomotor rhythmicity is confirmed by a delay in PER and TIM clock proteins cycling in the s-$LN_v$s, which is known to direct locomotor behaviour in constant darkness conditions (**Fig.15** and **Fig.16**). Flies were entrained for at least 4 days in controlled 12h light:12h dark conditions and then let free-run in constant darkness. Adult fly brains were dissected every 4 hours from the dark part of the last day of LD (ZT12) to the half part of DD2 (CT36). Anti-PDF immunostaining was used to reveal the small and large lateral neurons, which are then easily distinguished according to their size and localization.

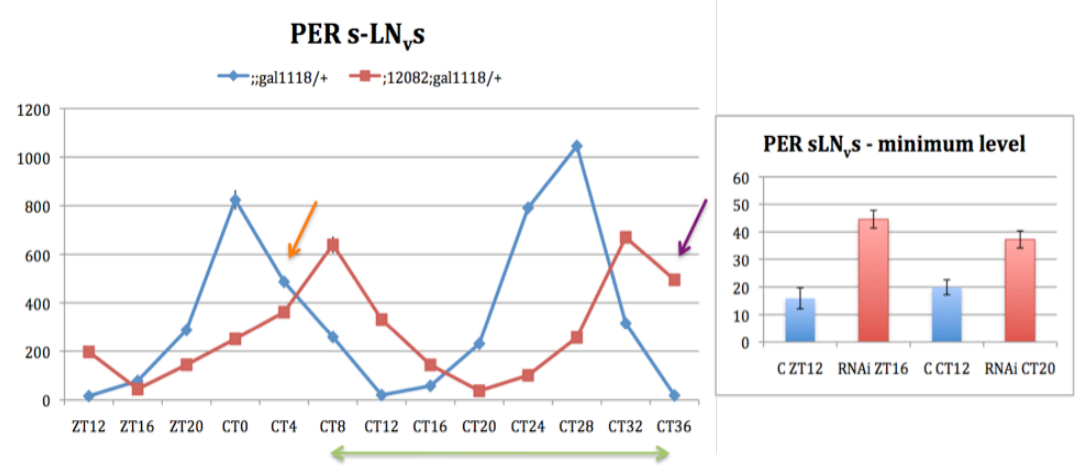



**Fig.15** PER cycling in the s-LN$_v$s from the end of the last day of LD (ZT12) to the first half part of DD2 (CT36). For each time point a vertical bar is used to show standard deviation errors. A green double-headed arrow indicates the period of mutant flies (tau= 28h). We suspect we've had some problems in the quantification of PER protein in control flies at CT4 (which normally corresponds to the maximum pick) (orange arrow), and at CT36 for the mutants (violet arrow), which are expected to be higher in both cases. The panel show a histogram of the minimum protein level found in both *usp5*-RNAi (RNAi) flies and in control (C) flies, with the respective times. X-axis: time; Y-axis: average fluorescence of neuron cells normalized with background fluorescence.

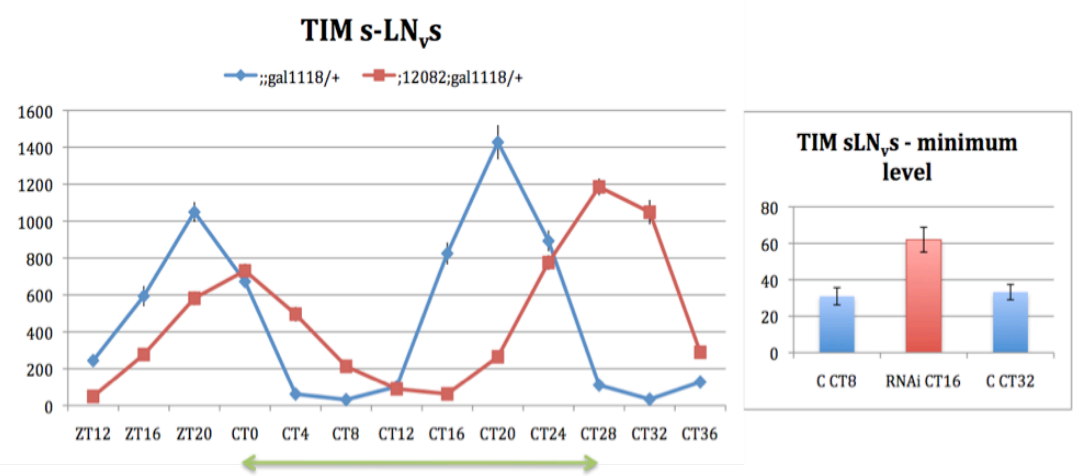



**Fig.16** TIM cycling in the s-LN$_v$s from the end of the last day of LD (ZT12) to the first half part of DD2 (CT36). For each time point a vertical bar is used to show standard deviation errors. A green double-headed arrow indicates the period of mutant flies (tau= 28h). The panel show a histogram of the minimum protein level found in both *usp5*-RNAi (RNAi) flies and in control (C) flies, with the respective times. X-axis: time; Y-axis: average fluorescence of neuron cells normalized with background fluorescence.

Clearly, in RNAi expressing flies, both TIM and PER proteins cycle with a period lengthened of about 4 hours (tau=28h), compared to that displayed by control flies (tau=24h). Moreover, RNAi flies show a lower amplitude of the cycle. The analyzed clock proteins fail to accumulate to their expected maximum level and interestingly, it seems that they could not even reach their minimum quantity (see histograms of **Fig.15** and **Fig.16**), indicating a certain accumulation when proteins are normally degraded.

To test if and how protein cycling is perturbed during light:dark cycles, the analysis was performed again with the same experimental conditions, but including flies collection for all the last day of LD entrainment. During the previous experiment we found some morphological defects in the lateral neurons of RNAi flies (aberrant size) that, we suppose, are caused by the strong RNAi expression. We thus decided to use a different genotype, which displays a slightly less strong phenotype when tested for locomotor free-running activity (the period length – tau, is of 28 hours instead of 29 hours). The following graphics (**Fig.17** and **Fig.18**) show the data obtained from PER and TIM protein quantification during LD conditions.

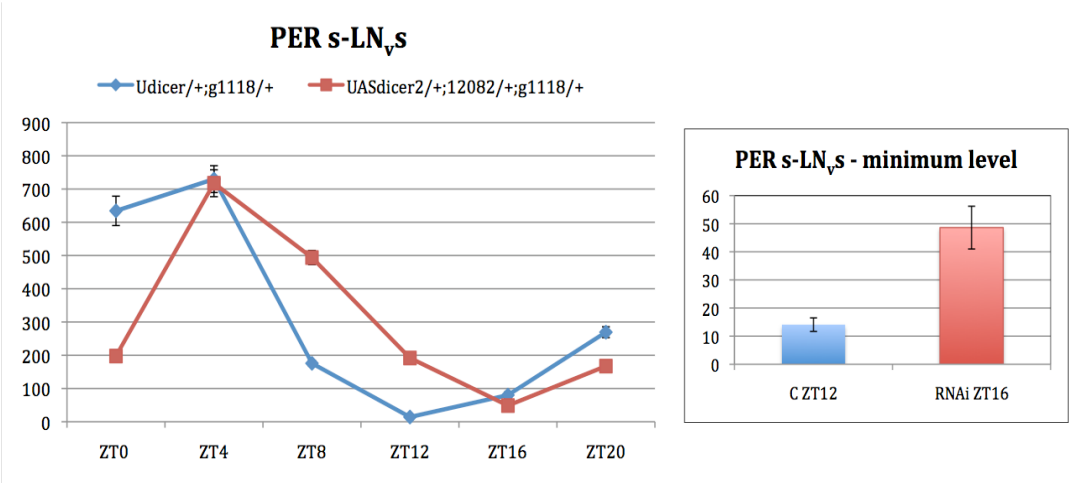



**Fig.17** PER cycling in the s-LN$_v$s during standard LD conditions. For each time point a vertical bar is used to show standard deviation errors. The panel show a histogram of the minimum protein level found in both *usp5*-RNAi (RNAi) flies and in control (C) flies, with the respective times. X-axis: time; Y-axis: average fluorescence of neuron cells normalized with background fluorescence.

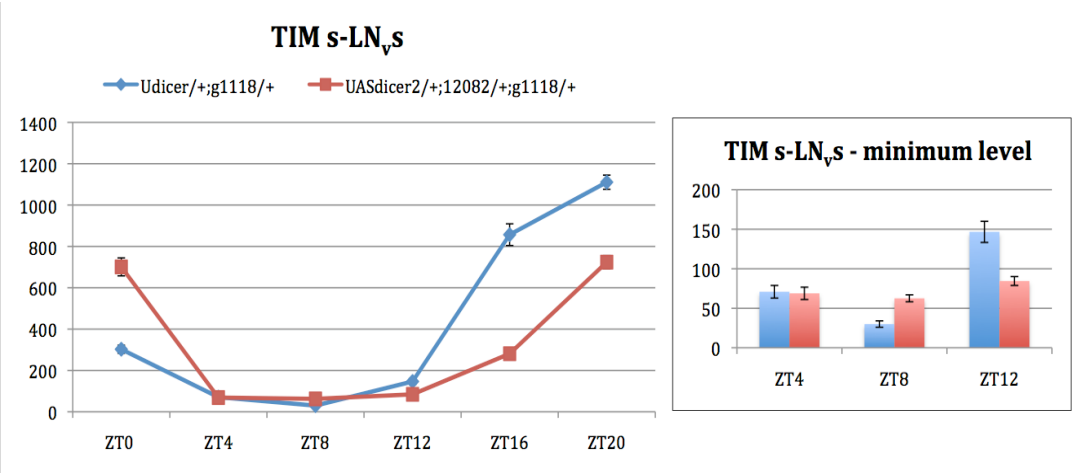



**Fig.18** TIM cycling in the s-LN$_v$s during standard LD conditions. For each time point a vertical bar is used to show standard deviation errors. The histogram in the panel show in a large-scale the quantity of TIM in both *usp5-RNAi* flies (red bars) and in control flies (blue bars) during their minimum level time points. X-axis: time; Y-axis: average fluorescence of neuron cells normalized with background fluorescence.

The quantification results of the last part of the LD are strongly comparable between the two different experiments, hence validating the reliability of the data obtained. In the presence of light, PER degradation is shifted of about 4 hours in *usp5*-RNAi flies, and its accumulation does not seem perturbed (we wonder if the

maximum peak displayed is truly as high as the control one). Both for control and tested flies, TIM degradation remains strongly dependent from the light input, during which TIM protein level in RNAi-flies falls with a curve that reproduces the one shown by the controls. Nevertheless, TIM accumulation in mutants is slowed down and seems to persist a little bit more after the lights-on.

In control flies, TIM and PER proteins cycling is highly similar in LD and DD conditions. For transgenic RNAi-expressing flies we can observe that: (1) For both tested conditions, the amplitude of the cycle is reduced: proteins fail to be degraded to the same level shown in controls, and can not even reach their respective maximum quantities (peak). (2) TIM degradation is clearly slowed-down in the absence of the light-stimulus. As the curve of TIM accumulation is comparable to that displayed by the control flies, we can hypothesize that the delayed degradation in DD conditions accounts for the period's shift. (3) PER degradation's curve appears quite similar in LD and DD conditions, with a delayed accumulation that is slower during constant darkness. Hence, PER delayed cycling appears caused by a shifted in the accumulation time. Nevertheless, the results shown so far have been obtained only once; these primary observations have thus to be confirmed (especially for PER).

## Analysis of the *usp5* P-insertional mutant

Even after multiple cycles of out-crossing, we were not able to find any homozygous flies in the mutant line carrying the P-element insertion in *usp5*gene (line #22426). An attentive analysis reveals that the homozygous flies do hatch with larvae starting eating and growing until the 3$^{rd}$ instar (L3), after which they would normally enter the pupal stage. On contrary, *usp5* mutant larvae died as soon as L3. As Usp5 is an ubiquitin protease, its enzymatic activity can ostensibly be required for the proper turnover of molecules during development, which would explain the mutant lethality. RNA interference, on contrary, reproduces hypomorphic and cell-targeted effects, which explains the viability of *usp5*-RNAi

flies. We decided to study the viable mutant larvae. L3 mutant larvae have been first dissected to test for a normal clock neurons development, which has been confirmed by anti-PDF immunostaining (data not shown). Therefore, we analyzed the clock functioning in apparently healthy neurons. To do so, adult male and female flies heterozygous for the mutation in *usp5* gene have been kept 4 days in 12:12 h LD cycles, during which females have deposed eggs. Then, the tubes remained 3 days in constant darkness conditions. 3$^{rd}$ instar larvae were collected four times (on intervals of 6 hours) during the third day of DD (at which time we expected to see a stronger phenotype). Larval brains were treated with anti-PER, anti-TIM and anti-CLK immunostaining (in this case, we are not obliged to use an anti-PDF immunlabeling to identify target cells as the only clock neurons already formed during larval stages are the lateral and dorsal ones, easily distinguishable), and the protein levels quantified in the s-LN$_v$s (**Fig.19**).

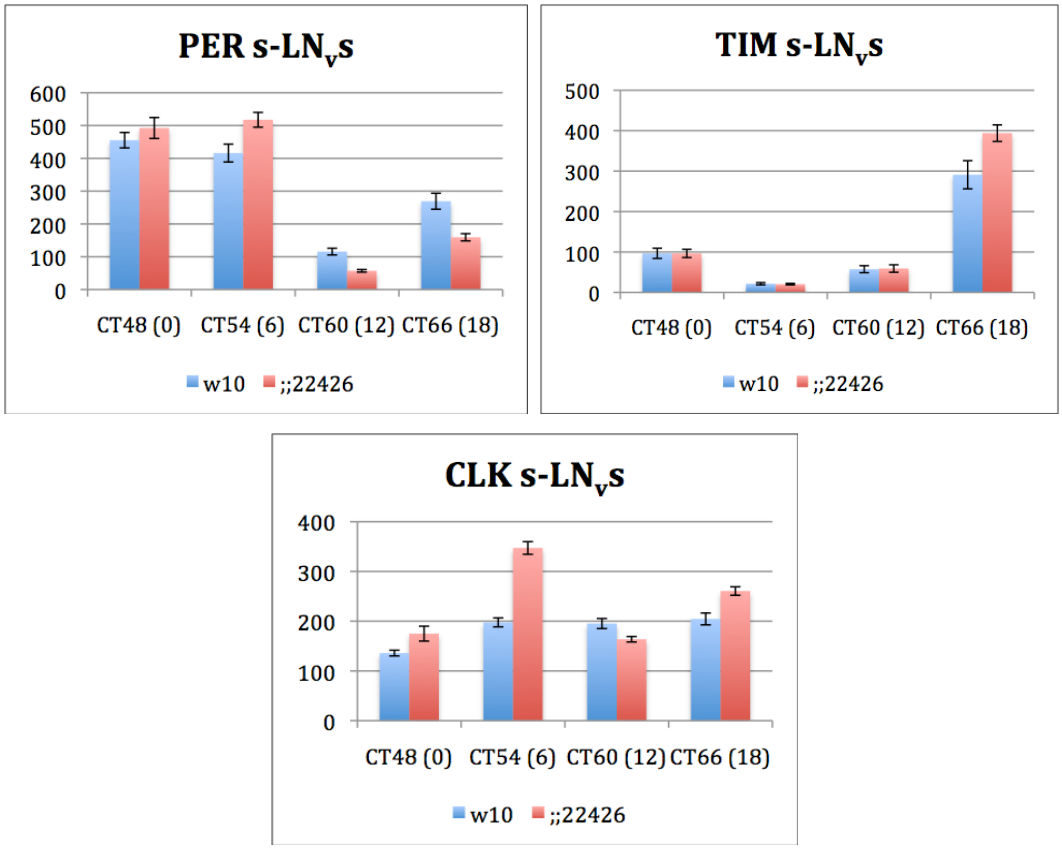



**Fig.19** Quantification of PER, TIM and CLK proteins in the s-LN$_v$s of larvae homozygous for the *usp5* mutation. ;;22426: mutants; w10: wild-type control flies. X-axis: time (on brackets, time referred to 24-h); Y-axis: average fluorescence of neuron cells normalized to the background one.

The analysis gave complex results. PER and TIM cycling appears not particularly modified. CLK protein cycling is generally rather difficult to be visualized (as, in fact, shown by controls) and we thus considered not so significant the strong difference between wild-type and mutants flies found in CLK protein quantification at CT54. Many aspects should be taken in account. First, the analysis is based on only four time-points, which could not be sufficient to describe the real effects of Usp5 absence on the clock protein oscillations. Second, we have to confirm that the insertion does effectively affect the *usp5* gene, and that other genes' expression is not altered by the insertion. In fact, *usp5* is flanked by genes that could possibly be required during development (genes coding for proteins involved in the biosynthetic process, DNA binding, etc.). Thus, the mutation of a gene different from *Usp5* could effectively be responsible for the lethal phenotype. As the P-element insertion in *usp5* seems to have no effects when carried only by one allele, a complementation test using another *usp5* mutation will be done to verify whether *usp5* is affected.

Is it possible to increase *usp5*-RNAi phenotype when the expression is driven by *timgal4*?

Different from the quantification data obtained by immunofluorescence, western blot analysis in free-running constant darkness conditions doesn't show a clear delayed and lengthened cycling of proteins. A reason is that the head extracts used for the western blot analysis derived from flies that display just 1.5 hours lengthening of the period, whereas brains analyzed for protein cycling by immunofluorescence derived from flies that behave with a 28/29-hours' period. A stronger phenotype with *timgal4* driver is thus required. Moreover, long-period *timgal4*/RNAi flies are searched to attest Usp5 function in controlling behavioural outputs in LD conditions. To enhance *timgal4* phenotype we decided to use the same protocol defined for the putative partners of CRY: co-overexpression of Dicer2. *timgal4*/UASdicer2 flies were crossed with *usp5-RNAi* flies and the male progeny tested for locomotor rhythmicity in constant dark conditions for 10 days,

after entrainment in LD. The test was initially performed at 25°C. As some genotypes appear lethal in these conditions, we tried to reduce the transgenic expression performing the same crosses at a lower temperature (20°C). **Table7** shows the most significant results obtained.

| **Genotype** | **Test T°** | **Alive flies (tested)** | **% R** | **Tau (sem)** | **Power (sem)** |
|---|---|---|---|---|---|
| ;*timgal4*/12082$^{NIG}$;UASdicer2/+ | 25°C | 15 (16) | 80,0% | 28,8 (0,26) | 43,9 (7,68) |
| ;*timgal4*/12082$^{NIG}$; | 25°C | 16 (16) | 100% | 24,2 (0,08) | 111,8 (11,89) |
| ;*timgal4*/+;UASdicer2/+ | 25°C | 16 (16) | 75,0% | 24,2 (0,11) | 110,0 (15,16) |
| ;*timgal4*/12082$^{VDRC}$;UASdicer2/+ | 20°C | 16 (16) | 75,0% | 29,3 (0,95) | 67,3 (10,76) |
| ;*timgal4*/12082$^{VDRC}$; | 20°C | 15 (16) | 100% | 23,9 (0,06) | 210,5 (15,18) |
| ;*timgal4*/+;UASdicer2/+ | 20°C | 16 (16) | 93,8% | 23,6 (0,10) | 220,6 (0,51) |

**Table7** Locomotor activity of flies co-expressing *usp5*-RNAi, *timgal4* and UASdicer2. The first column describes the phenotype tested. The second column reports the temperature at which the test has been performed. Subsequently is presented the number of flies tested and the rhythmicity shown by the flies that live until the end of the experiment (alive flies), with their period and the robustness of the rhythm (power). *timgal4*/UASdicer2 flies are taken as negative reference for the periodicity. The tests without the UASdicer2 construct are used to compare the effect of dicer2 overexpression.

The behavioural data confirm that overexpression of Dicer2 acts to increase the RNAi phenotype. These results give us new, more favorable conditions to test Usp5 down-regulation effects using western blot and by locomotor activity in LD (eduction).

# CONCLUSIONS AND PERSPECTIVES

The clock is a complex machinery whose wheels have to be both strictly settled (period) and highly easy to change (phase) at the same time. The recent years have provided important progress in the comprehension of the clock although a lot of pieces are still missing.

An important and not completely solved question is to understand how the light stimulus is molecularly perceived in order to reset the clock. We tried to answer analyzing putative partner proteins of CRY in constant light conditions. Our study suggests that the proteins tested are not strong modulators of the CRY light-stimulus transducing to the brain clock. Another interesting possibility is that the candidate proteins can effectively interact with CRY but in a way that do not alter the locomotor rhythmic behavior, but still could modify other clock-controlled responses or clock-independent activities. For example, it has been shown that in peripheral tissues CRY plays a role of a transcriptional repressor (Ivanchenko M. et al., 2001). As locomotor behaviours are driven by the brain clock, the possibility of our proteins' involvement in the timekeeping machinery of peripheral tissues can not be appreciated using a locomotor activity rhythm analysis and has to be tested trough different approaches. Yeast two-hybrid system is a heterologous method whose results can be altered by numerous false positives, i.e. the interactions found would not occur in the real cellular environment. However, other approaches must be tested to validate the lack of involvement of these genes in CRY activity or stability. For example, we could test CRY putative interactors' activity in the eyes. The optomotor test is a classical protocol used to verify the fly's capacity to perceive the movement. We've defined a sub-group of the most interesting putative CRY partner proteins, and flies expressing the respective RNAi are in program to be tested using this approach.

Ubiquitin specific peptidase 5 (USP5) is a new protein involved in the regulation of the clock ticking. Locomotor activity data have shown that the protein is required for proper 24-hours periodicity in free running condition, while its down-regulation induces a slow-down of clock protein cycling and a long-period phenotype. Quantification data obtained by western blot analysis of fly head extracts and immunofluorescence in whole-brains are consistent with a delayed clock proteins accumulation and a dysfunction in the degradation process. These effects are particularly evident in total darkness, which is consistent to a specific activity of Usp5 in free running conditions and not in light-mediated protein degradation. An interesting, still unsolved question is to understand if Usp5 activity is specific or rather general. We've noticed that *usp5* down-expression correlates with a stabilization of the active forms of CLK, which may lead to a higher transcription of CLK-controlled genes, thus an increase of PER, TIM, VRI and PDP1epsilon proteins quantities. A restricted effect of Usp5 on CLK protein can solely explain these proteins' accumulation. To verify the possibility of an enhanced CLK activity, the quantification of the amount of CLK-controlled genes' mRNAs by RT-PCR is in progress. Preliminary results suggest that transcription is not increased. As it is an ubiquitin protease, Usp5 could have an important role in post-translational protein modifications by controlling ubiquitin-targeted degradation. A simple way to check whether Usp5 is involved in post-translational modification is to test $per^{0}$/*UAS-per16* flies for rhythmic behaviour in constant darkness. In these flies, PER protein expression is not controlled by the clock but by the driver *gal4* transgene, and it is thus constitutive. As they display normal rhythmic locomotor behaviours, these must have been induced by post-translational modification in proteins, which controls rhythmic outputs. If Usp5 is somewhere involved in post-translational modifications that controls protein cycling, *usp5*-RNAi should render flies arrhythmic, while it has no impact if Usp5 controls the transcriptional event. Moreover, as we are talking about a cycle, Usp5 activity could be restricted to a single protein, which in turn controls other protein quantities, or a-specific. To see whether this dependence exists, we will test different genetic backgrounds, mutants for a single clock protein, and check *usp5*-RNAi effects in the other proteins.

Another important point is to define Usp5 enzymatic function, which has not yet

been characterized in *Drosophila*. Nevertheless, *Drosophila usp5* homologs in yeast (Ubp14) and mammals (USP5/IsopeptidaseT) have shown to possess an important role. *Drosophila* Usp5 and human IsopeptidaseT (hUsp5/IsoT) show the same domain organization and, moreover, a high primary sequence similarity. According to this, we could postulate that *Drosophila*'s USP5 may act (at least) in the disassembly of free poly-ubiquitin chains (see Introduction). This activity is very important for a cell, as it is shown that free ubiquitin polymers actively compete with Ub-tagged proteins for proteasome recognition thus blocking protein degradation. Usp5 down-regulation thus is expected to induce the accumulation of ubiquitinated proteins. This correlates with the result obtained from western blot analysis, where proteins are shown to accumulate (in DD) at those times they would normally be degraded. Nonetheless, a "classical" role of Usp5 in antagonizing ubiquitin-ligases activity is not *a priori* excluded. Reversible ubiquitination of proteins is indispensable to maintain the correct balance into polypeptides accumulation and destruction. The loss of Usp5 activity would thus favor the degradation of ubiquitin-tagged proteins. An abnormal destruction can explain the slower accumulation and the incapacity of proteins to reach their maximum pick level as shown in *usp5-RNAi* brains by clock proteins immuolabeling. We can take advantage of an immortalized culture of *Drosophila* embryo cells (S2 line) to analyze clock proteins ubiquitination as well as the levels of free poly-ubiquitin chains in the presence of *usp5* RNAi. Anti-Usp5 antibodies or transgenes encoding a tagged Usp5 polypeptide can be used to analyze its subcellular localization and/or its involvement in clock protein complexes by co-immunoprecipitation.

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