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# Functional analysis of circadian clock gene *timeless* in temperature compensation mechanism

Ph.D. Thesis

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## Annotation

This thesis is focussed on investigating the role of the circadian clock gene *timeless* in temperature compensation mechanism in *Drosophila melanogaster*. To conduct the studies presented in this thesis advanced gene editing technique, CRISPR/Cas9, was used to target *timeless* and to create a variety of mutations. Application of various reverse genetics tools and behavioural methods described here contributed to the elucidation of *timeless* role in temperature compensation of the circadian clock.

## Declaration

I hereby declare that I did all the work presented in this thesis by myself or in collaboration with co-authors of the presented papers. I properly cite all references and other sources that I used to work up the thesis. Those references and other sources are given in the list of references.

České Budějovice — Samarjeet Singh

09/04/2020

## Declaration [in Czech]-Prohlášení

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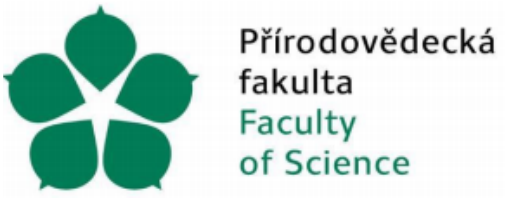



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I dedicate this work to all of you.

## List of papers and author's contribution

This thesis is based on the following papers:

**Singh S,** Giesecke A, Damulewicz M, Fexova S, Mazzotta GM, Stanewsky R, Doležel D. (2019) New drosophila circadian clock mutants affecting temperature compensation induced by targeted mutagenesis of *timeless*. *Front Physiol.* 3; 10:1442.
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**Samarjeet Singh** participated in the design of the study, performed majority of the experiments, data analysis, interpreted the results and contributed to writing of the manuscript.

**Singh S**, Doležel D. Temperature dependent splicing in the regulation of circadian rhythms in *Drosophila melanogaster*. Unpublished results
**Samarjeet Singh** participated in the design of the study, performed all experiments, data analysis, interpreted the results.

**Singh S**, Doležel D. Exploring the unexplored role of *timeless* in temperature compensation and light sensitivity. Unpublished results
**Samarjeet Singh** participated in the design of the study, performed all experiments, data analysis, interpreted the results.

## Co-author agreement

Dr. David Doležel, the supervisor of this Ph.D. thesis and co-author of all presented papers, fully acknowledges the contribution of Samarjeet Singh

David Doležel

# Table of Contents

List of Abbreviations……... 1
Thesis overview……... 4
Introduction
1.1 Circadian rhythms…… 7
1.2 Significance of circadian rhythms…… 8
1.3 *Drosophila*- a model for studying circadian rhythms…….. 10
1.4 Locomotor activity rhythms…… 11
1.5 Molecular basis of circadian rhythms…….. 13
1.6 Circadian clock and Temperature Compensation…… 17
1.7 Advantages of using *Drosophila melanogaster*…….. 20
1.8 CRISPR/Cas9 system……...20
1.9 Aim of the thesis…….. 22
1.10 References…… 23

Chapter 1
New *Drosophila* circadian clock mutants affecting temperature compensation induced by targeted mutagenesis of *timeless*
1. Introduction..... 40
2. Material and methods..... 41
3. Results
   3.1 Genetic interaction between *tim, cry*, and *per*…… 44
   3.2 $tim^{blind}$ is a temperature compensation mutant…… 45
   3.3 Step-up protocol for detection of temperature compensation mutants…… 47
   3.4 CRISPR/CAS9 targeted mutagenesis of *tim*……... 47
   3.5 Functional characterization of novel mutant lines…….. 49
   3.6 Immunocytochemistry…… 53
4. Discussion..... 56
5. References..... 59
6. Supplementary material..... 62

Chapter 2
Temperature dependent splicing in the regulation of circadian rhythms in *Drosophila melanogaster*
1. Introduction..... 74

# List of Abbreviations

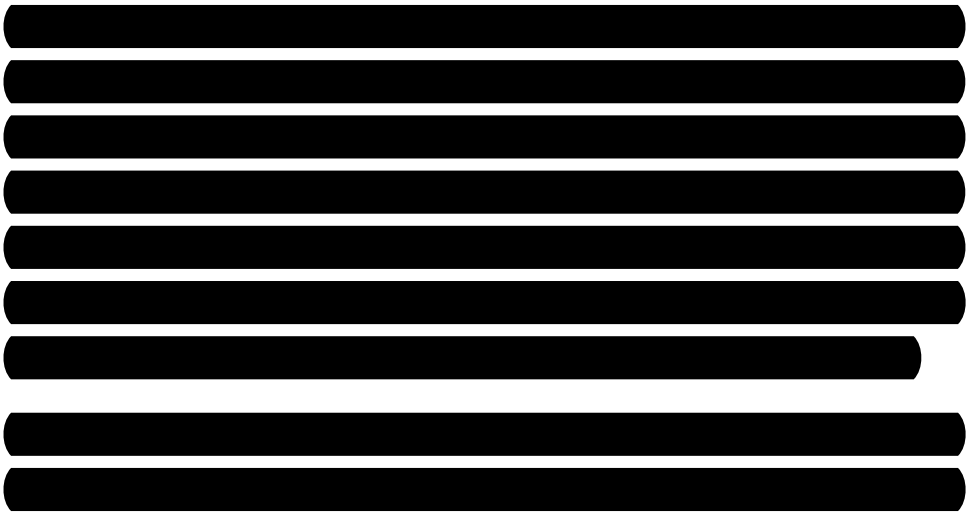
| | |
|---|---|
| AS | alternative splicing |
| bHLH | basic-helix-loop-helix |
| Cas | CRISPR Associated system |
| CK1 | casein kinase 1 (protein) |
| CK1ε/δ | CASEIN KINASE 1ε/δ (proteins) |
| CK2 | casein kinase 2 (protein) |
| CLD | cytoplasmic localization domain |
| *Clk,* CLK | *Clock* (gene, transcript), CLOCK (protein) |
| CRISPR | clustered regularly interspaced palindromic repeats |
| crRNA | CRISPR RNA |
| *cry*, CRY | *cryptochrome* (gene, transcript), CRYPTOCHROME (protein) |
| *cyc,* CYC | *cycle* (gene, transcript), CYCLE (protein) |
| DAM | *Drosophila* activity monitoring system |
| DAM2 | *Drosophila* activity monitoring system 2 |
| DBT | DOUBLETIME (protein) |
| DD | constant darkness |
| DSB | double strand break |
| dsDNA | double-stranded DNA |
| eGFP | enhanced green fluorescent protein |
| EMS | ethyl methane sulfonate |
| ESE | exonic splicing enhancers |
| FRP | free-running period |
| GSK3 | glycogen synthase kinase 3 (protein) |
| hnRNP | heterogeneous nuclear RiboNucleoProteins |
| HR | homologous recombination |
| ICC | immunocytochemistry |
| JET | JETLAG (protein) |
| $jet^c$ | particular allele of *jetlag* gene |
| LD | light/dark |
| LL | constant light |
| mRNA | messenger RNA |
| NES | nuclear export signal |
| NHEJ | non-homologous end joining |
| NLS | nuclear localization signal |

| | |
|---|---|
| PAM | protospacer adjacent motif |
| PAS | Per-Arnt-Sim |
| PCR | Polymerase chain reaction |
| *Pdp1,* PDP1 | *PAR domain protein 1* (gene, transcript) and PDP1 (protein) |
| *per*, PER | *period* (gene, transcript) and PERIOD (protein) |
| PP2A | protein phosphatase 2A (protein) |
| SGG | SHAGGY (protein) |
| SLIMB | supernumerary limbs (protein) |
| snRNA | small nuclear RNA |
| snRNP | small nuclear RiboNucleoProtein |
| TBE | Tris-borate-EDTA |
| *tim*, TIM | *timeless* (gene, transcript) and TIMELESS (protein) |
| tracrRNA | trans-activating crRNA |
| UTR | untranslated region |
| V/P | VRI/PDP1ε |
| *vri,* VRI | *vrille* (gene, transcript) and VRILLE (protein) |

## Thesis overview

This thesis consists of three chapters, each dealing with the goal to explore the connection of circadian clock gene *timeless* (*tim*) and temperature compensation mechanism. The beauty of this mechanism relies on the insensitivity of period to changes in temperature and any defect in this process is responsible for disturbance in organism's sleep–wake pattern including other physiological processes, proposing the relevance of the studies conducted in this thesis.

In the **first chapter**, in an attempt to reveal the mechanisms underlying temperature compensation, 7 regions in *tim* were sequentially targeted by CRISPR/Cas9 based on sequence conservation across species. This study produced 20 unique mutants which identified a 150 bp (50 amino acids) region of *tim* that is particularly important for the functionality of temperature compensation mechanism. These data are contributing insights into the field of molecular chronobiology in general and are important for research on TIM protein in particular.



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# Introduction

## Circadian rhythms

The planet Earth pirouettes on its axis every 24 hours which results into imbuing of its surface in alternate sunlight and darkness cycles. These alternate changes produce variations in the physical properties of the environment such as light intensity (Pittendrigh, 1967), temperature (Glaser & Stanewsky, 2005; Liu, 1998; Pittendrigh, 1954; Yoshii et al., 2005) and humidity (Mwimba et al., 2018). Similarly, in response to these defined everyday changes in the environment most organisms also display daily changes in their physiological and behavioural processes such as sleep-wake cycles, oscillation in hormone levels, reproductive cycles and other important biological processes. These changes in the physiology and behaviour are not just a mere response to daily fluctuations in the environment, but arise from internal time keeping mechanisms referred to as circadian clock or rhythm (derived from Latin term *circa diēm*, meaning – about a day) which run with a periodicity of about 24 hours.

Circadian rhythms are characterized by three important features, which are as follows:

• **Endogenous**: Are self-sustained and persist with a period of approximately 24 hours even in the absence of any environmental cues.

• **Temperature compensated**: Maintain 24-hour periodicity over a range of physiological temperatures unlike other biochemical processes, which increase their rate with rise in temperature. It allows circadian clocks to precisely measure time despite day/night and seasonal changes in temperature.

• **Entrainable**: Can be synchronized and reset by various environmental time cues such as light, temperature, humidity, etc. This ability helps to synchronize the appropriate timing of behaviour and physiology with seasonally and geographically changing light–dark cycles and thus confers maximum survival advantage to the organisms.

**Significance of circadian rhythms**

In the history of our planet, while many things have changed but there has always been one constant thing which is day-night cycles. The reason for the existence of circadian rhythms is to provide a window to the organism to repair, reset and rejuvenate themselves in a daily manner. Secondly, circadian rhythms allow organisms to enhance their fitness by enabling them to efficiently anticipate periodic events such as availability of food, light, temperature and mates and thus help them to prepare themselves for these day-night cycle associated environment changes giving an advantage to survive under ever-changing environments. Thus, organisms which have circadian period similar to that of light-dark cycles have the competitive edge over others. For example, the rhythmic strains in cyanobacteria have enhanced reproductive fitness over arrhythmic strains when placed in a day-night cycle (Ouyang et al., 1998; Woelfle et al., 2004). Similarly, timing behaviour according to the external environment is also crucial for other organisms to avoid competition for food as well as for predation. DeCoursey et al. in their simulated field study on ground squirrels favoured rhythmic over arrhythmic for their susceptibility to predation (DeCoursey et al., 1997). Therefore, circadian rhythms help to timely adjust the behavioural and physiological changes

in the organisms that contribute to their fitness and survival in daily challenges in the environment.

Circadian rhythms are nearly ubiquitously present in organisms ranging from unicellular cyanobacteria to highly complex mammals such as human beings (Czeisler et al., 1999; Ouyang et al., 1998). Therefore, like other organisms, human physiology is also profoundly affected by the day-night cycles and it has immense implications on human health. Circadian dysfunction/desynchronization is a common problem in modern lifestyle of humans which is an outcome of many artificial and some natural reasons. First, day/night shifts and altering feeding patterns misalign the internal clock from the external time which results into negative health outcomes such as insomnia and increased risk of obesity, cardiovascular diseases, type-2 diabetes and cancer (Braunwald, 2012; Erren & Reiter, 2008; Lévi et al., 2000; Scheer et al., 2009; Spiegel et al., 2005). Another frequent problem because of desynchronized circadian clock is jetlag observed in travellers/pilots travelling across different time zones. It is associated with fatigue, sleep problems and takes few days until the circadian clock gets resynchronized to the local time (Barion & Zee, 2007). Among natural reasons are seasonal fluctuations in the environment which alters the circadian rhythms leading to mood disorders, winter depression (Lewy et al., 2006). Hence, circadian rhythms are universal to diverse life on this planet in different aspects. It is therefore important to extend our basic understanding of the mechanisms regulating circadian rhythms and its synchronization to the environment could help us to develop treatments dealing with the dysfunctional circadian clocks and its subsequent effect on health and fitness as a whole.

## *Drosophila*- a model for studying circadian rhythms

*Drosophila melanogaster* commonly known as fruit fly has served as an excellent genetic model to decipher molecular basis of circadian clock. A variety of behaviours in *D. melanogaster* are known to be regulated by circadian clock such as - eclosion (emergence of adult fly from pupa) (Konopka & Benzer, 1971), period of rest and activity, olfactory sensitivity (Krishnan et al., 1999), egg laying (Manjunatha et al., 2008), courtship (Fujii & Amrein, 2010; Fujii et al., 2007), gustatory sensitivity (Chatterjee et al., 2010) and learning and memory (Lyons & Roman, 2009).

It was Colin Pittendrigh, one of the founding fathers of modern Chronobiology, who first used *Drosophila* as a model organism for circadian rhythm research such on topics as entrainment and temperature compensation. In his study, he used various species of *Drosophila* to demonstrate that a true biological clock controls the eclosion, which peaks around dawn. His experiments also showed that the circadian clock is endogenous, entrainable and temperature compensated (Pittendrigh, 1954, 1993).

The first circadian gene – *period* (*per*), which is conserved in the clocks of animals including mice and humans (Sun et al., 1997; Tei et al., 1997; Zylka et al., 1998) was identified in *Drosophila*. Seymour Benzer and his student Ronald Konopka (Konopka & Benzer, 1971) identified *per* in a screen for flies with abnormalities in eclosion rhythms after a chemical mutagenesis induced by ethyl methane sulfonate (EMS). In their screen they isolated three mutant strains - $per^{L}$ (longer period of 29

hours), *per*$^{S}$ (shorter period of 19 hours) and *per*$^{0}$ (no rhythm in eclosion-arrhythmic) (Konopka & Benzer, 1971).

## Locomotor activity rhythms

Locomotor activity is a very robust behavioural read out of circadian rhythms. In *Drosophila*, it is monitored by placing individual flies (1-4 day old) in a small glass tubes with food at one end and a

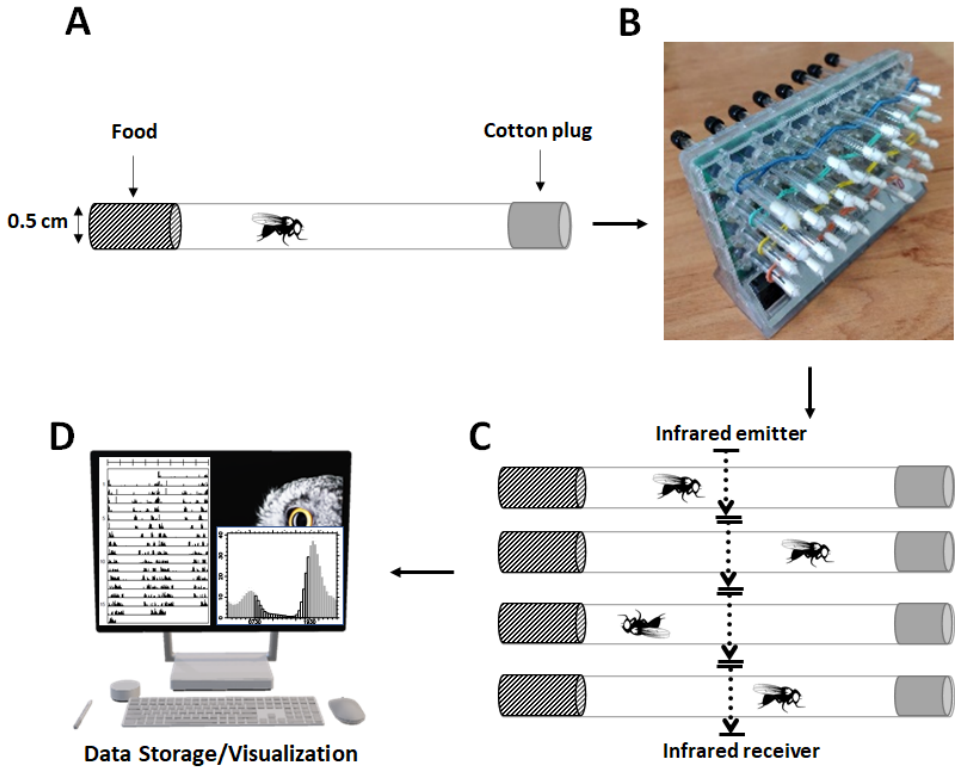


**Figure 1. Locomotor activity rhythms monitoring assay in *Drosophila*.** (A) Individual flies are loaded into glass tubes (0.5 cm in diameter) with food at one end and a cotton plug at the other end. (B) These glass tubes are placed into *Drosophila* activity monitors and then are placed into an incubator (with controlled light, temperature and humidity conditions) connected to a computer. (C) Each time a fly moves in the tube and crosses the infrared beam, it is recorded by the computer. (D) Locomotor activity can be represented as a double-plotted Actogram (left) or histogram (right).

stopper at the other end (Fig. 1). These tubes are then loaded into locomotor activity monitors (Trikinetics Inc, Waltham, MA USA) and placed in incubators with controlled light, temperature and humidity conditions, connected to a computer equipped with *Drosophila* activity monitoring (DAM) system. The activity monitors are equipped with infrared emitters and receivers. An infrared beam, which is aimed at the centre of the glass tube, is broken every time the fly moves across the tube and recorded by DAM system (In our lab activity counts are saved in 5-minute bins) (Chiu et al., 2010; Rosato & Kyriacou, 2006).

In controlled conditions, locomotor activity is assessed by subjecting flies to 12:12 light-darkness (12:12 LD) which mimics a natural day-night cycle. Under 12:12 LD conditions, *D. melanogaster* shows two peaks of locomotor activity, one in the anticipation of lights-on (Morning (M) peak) and one 3-4 hours before the lights off (Evening (E) peak). The M and the E peaks of activity are not merely a response to lights-on and lights-off respectively but suggest a bi-modal pattern of activity in a LD cycle under the control of circadian clock. This locomotor activity pattern under LD can be plotted as a function of time by a histogram.

To access the functioning of the endogenous clock, which persists in the absence of any stimuli, flies after entrainment in 3-4 days of 12:12 LD cycle are released into constant darkness (DD) for 10 days to characterize the free running clock. In DD conditions, wild-type flies generally produce a unimodal activity peak, which occurs approximately at the same time every day reflecting the 24-hour period length of locomotor activity rhythm commonly known as the free-running period

(FRP). This activity pattern can be represented by a double plotted daily activity plot known as Actogram (Fig. 2).

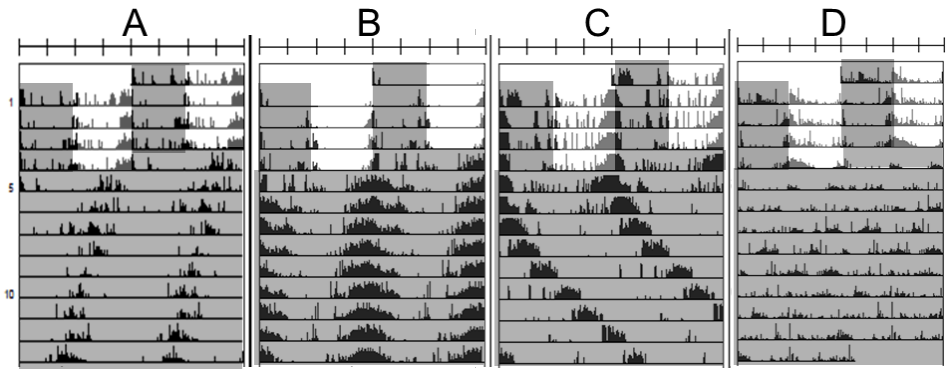


**Figure 2. Representative actograms of Wild-type (WT) and mutant flies with different free-running periods (FRP)**. (A) Period length shorter than 24 hours. (B) Period length almost equal to 24 hours (Wild-type FRP). (C) Period length longer than 24 hours. (D) An example of arrhythmicity (non-functional clock). Light regimes (white: light; black: constant dark).

Actogram is a plot with the time of the day on x-axis and number of days in an experiment on the y-axis. In a double plotted actogram, each day is plotted twice, first on the right half and then on successive line on the left half of the plot. In a double plotted actogram, the period length shorter than 24 hours can be deduced from the activity peaks drifting towards the left (Fig. 2A) but on the other hand the activity peaks of long period flies drifts towards the right on successive days of the record (Fig. 2C).

**Molecular basis of circadian rhythms**

In *D. melanogaster*, circadian clock keeps the time via timely fluctuations in the clock related proteins in interdependent transcription-translation feedback loops synchronized in a 24hr time scale. Its core feedback loop consists of *Clock* (*Clk*) (Allada et al., 1998) and *cycle* (*cyc*)

(Rutila et al., 1998) genes which belong to basic-helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) domain containing transcription factor family. CLK and CYC proteins heterodimerize and around mid-day bind to E-box sequences (CACGTG) in the promoter of *per* (Konopka & Benzer, 1971) and *timeless (tim)* (Myers et al., 1995; Sehgal et al., 1994) to initiate their transcription in a timely manner (Darlington et al., 1998). These *per* and *tim* mRNAs are then translated into proteins, which accumulate in the cytoplasm, form heterodimeric complexes, and translocate into the nucleus. Once inside the nucleus they bind to CLK-CYC, inhibiting their binding affinity for the *per* and *tim* E-boxes, thereby negatively regulating the transcription of their own mRNAs (Fig. 3).

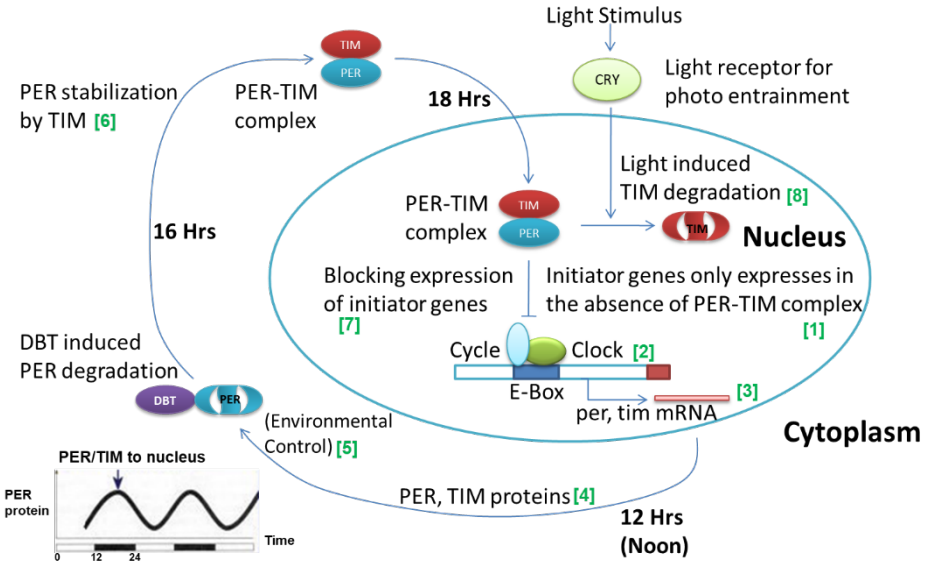


**Figure 3. A general overview of *Drosophila* circadian clock.**

In parallel to this core feedback loop, a second feedback loop exists which controls *Clk* transcription levels. During late day to early night when PER/TIM starts accumulating in the cytoplasm, CLK-CYC heterodimers also binds to E-box in the promoter of two additional circadian clock genes – *vrille (vri)* and *PAR domain protein 1(Pdp1)* (Blau & Young, 1999; Cyran et al., 2003; McDonald et al., 2001). VRI is a repressor and PDP1 is an activator of *Clk* transcription (Cyran et al., 2003; Glossop et al., 2003). VRI accumulate first (~3-6 hours earlier than PDP1) in phase with its mRNA levels and binds to VRI/PDP1ε (V/P) regulatory element in the promoter region of *Clk* gene to inhibit its transcription. Later, PDP1 accumulates in a delayed manner and high PDP1 levels compete out VRI from V/P region and promote *Clk* transcription. Hence, this loop brings greater stability to the core feedback loop.

To keep circadian time, the molecular events within the core feedback loop needs to be completed in ~24 hours. However, the various molecular processes within the cell including transcription, translation and repression collectively take much less than 24 hours to complete. Therefore, to extend the duration of all loops the mRNA levels of *per* and *tim* peak early in the evening, but the protein levels do not peak until late in the night and their proteins levels remain constantly low until dusk. This lag in the rise of protein levels is because of phosphorylation of PER by a kinase, DOUBLETIME (DBT) (Price et al., 1998), a mammalian ortholog of CASEIN KINASE 1ε/δ (CK 1ε/δ) (Kloss et al., 1998). These phosphorylations make PER prone to degradation by SLIMB, an E3 ubiquitin ligase (Grima et al., 2002; Ko et al., 2002). At the same time PER also get stabilized by protein phosphatase 2A (PP2A)

(Sathyanarayanan et al., 2004). At dusk, TIM slowly accumulates in the cytoplasm and physically associates with PER and stabilizes the phosphorylated PER (Gekakis et al., 1995; Vosshall et al., 1994). Hence, PER/TIM dimer accumulates in the early night and then translocates into the nucleus upon SHAGGY (SGG) dependent phosphorylation of TIM and CKII mediated phosphorylation of PER (Lin et al., 2002; Martinek et al., 2001) in an orchestrated manner several hours later. Once inside the nucleus, the PER/TIM complex inhibits their own gene transcription by reducing CLK-CYC heterodimer's binding affinity for E-boxes in the *per* and *tim* promoter (Darlington et al., 1998; Lee et al., 1999; Menet et al., 2010; Yu et al., 2006).

In the early morning, light causes activation of a photoreceptor CRYPTOCHROME (CRY) (Emery, 1998; Stanewsky et al., 1998) which changes its conformation and binds with TIM to direct it to a proteosome-dependent ubiquitin-mediated degradation via F-box protein JETLAG (JET) (Peschel et al., 2009). After TIM degradation, PER becomes unstable and prone to phosphorylation by DBT and ultimately also gets degraded. Absence of PER/TIM lifts the repression from CLK-CYC binding to the E-boxes and begins a new round of *per* and *tim* transcription. Thus, the clock resets to start an entire new cycle which takes about 24 hours to complete and hence generates circadian rhythms.

In addition to PER, proteins such as CLOCKWORK ORANGE (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008) whose transcription is itself regulated by CLK/CYC, have been shown to regulate CLK mediated transcription by competing CLK-CYC

binding to the E-box sequences in both *per* and *Clk* feedback loops and therefore provide greater precision to the molecular pacemaker.

**Circadian clock and Temperature Compensation**

In circadian biochemical system, despite of changes in ambient temperature, the period length remains essentially constant at approximately 24-h. The pioneering work of Colin Pittendrigh demonstrated that the *Drosophila* rhythm of eclosion (emergence of the adult fly from the pupa) retained a 24-h rhythmicity in total darkness over a temperature range of 16–26°C (Pittendrigh, 1954). This unique circadian biochemistry in *Drosophila* is thus an outcome of an unknown mechanism which most other biochemical reactions are lacking and consequently gets affected by temperature. This mechanism is later referred to as temperature compensation (Hastings & Sweeney, 1957; Pittendrigh, 1954).

Despite an important property of circadian clock, temperature compensation remained poorly understood mainly because of lack in the knowledge of all mechanisms that set the 24-hr circadian period. Therefore, through different mutations in circadian genes, efforts have been made to understand the role of these genes in determining the pace of clock. These studies identified mutants in the core clock genes which change the speed of the clock and run faster or slower but remains temperature compensated (ex. $tim^{UL}$ (Myers et al., 1997; Rothenfluh et al., 2000); $tim^{L1}$, $tim^{L2-}$ (Rothenfluh et al., 2000)). Further, various studies ($per^{L}$, $per^{S}$ (Ewer et al., 1990; Konopka et al., 1994; Konopka et al., 1989); $per^{SLIH}$ (Hamblen et al., 1998); $tim^{rit}$ (Matsumoto et al., 1999);$tim^{BLIND}$ (Wülbeck et al., 2005); $dbt^{S}$, $dbt^{L}$ (Price et al., 1998))

reported mutations in these genes responsible for altered periodicity with increase in temperature. Therefore, with the help of these mutants some period-determining reactions are known in *D. melanogaster* and out of them the first crucial step is the delay in peak expression of *per*/*tim* mRNA and protein levels which is around 6 hr in constant darkness in wild-type fly and is subjected to mRNA stability and processing regulated in a timely manner. Secondly, stability of PER and TIM in the cytoplasm and their subsequent nuclear translocation in the form of a dimer mainly relies on phosphorylation involving the enzymes casein kinase 2 (CK2), shaggy (a glycogen synthase kinase (GSK3) homolog), DBT (a casein kinase 1 (CK1) homolog). Even though these steps provided insights into the temperature compensation mechanism, the knowledge is still fragmentary, and the complete picture is unknown.

Additionally, daily oscillations in the levels of PER and TIM proteins are known to be modulated by temperature, RNAs oscillating at 18°C display earlier phases than those cycling at 25 or 29°C which advances the evening peak at 18°C (Majercak et al., 1999; Martin Anduaga et al., 2019). It is validated by a study confirming enhanced splicing of an alternative exon located in *per* 3' untranslated region (UTR) at low temperatures leading to higher levels of *per* mRNA and this advance in the mRNA level causes a phase advance in TIM and PER protein levels as well (Majercak et al., 1999). Despite these findings, it is still not clear how the regulation of the 3' UTR splicing impacts PER levels which is later known known to be regulated not only by temperature but also by the photoperiod (Collins et al., 2004). On the other hand, there is a reciprocal correlation of temperature and the amount of *per* and *tim* mRNAs where the abundance of *per* mRNA

increase at 20°C but that of *tim* mRNA decrease when compared to 25°C (Goto & Denlinger, 2002).

It complements the recent studies which showed that temperature changes greatly impact the pattern of alternative splicing of *tim* mRNA but not any other clock gene (Martin Anduaga et al., 2019), adding one more layer that it is not only light which regulates TIM, temperature also regulates *tim* by influencing its splicing pattern in an interesting way. Temperature influences the splicing pattern of *tim* at 18°C where it induces two cold-specific splicing isoforms, *tim-cold* (Boothroyd et al., 2007; Wijnen et al., 2006) and *tim-short&cold* (Martin Anduaga et al., 2019), but only *tim-cold* is translated into protein. However, at 28°C retention of different intron results into a shorter isoform (*tim-tiny*) (Shakhmantsir et al., 2018) which is not known to be translated . These findings suggest that these isoforms regulates the levels of the canonical TIM protein by post-transcriptional and/or post-translational regulation by miRNAs in a temperature dependent way (Martin Anduaga et al., 2019) which advances the clock at low temperatures and delays it at high temperatures to adjust the clock to 24 hr period i.e. keeping the clock temperature compensated.

Interestingly, these studies reflect that most of the changes in *tim* splicing patterns are conserved across several *Drosophila* species and correlate well with the capacity of the species to adapt their activity to temperature changes. It suggests that *tim* is the connecting link between circadian clock and temperature compensation and therefore, an important candidate to study temperature compensation mechanism.

**Advantages of using *Drosophila melanogaster***

*D. melanogaster* offers many advantages to geneticists due to its small size (adults are a few mm long), high fecundity rate (hundreds of progenies from a single female), rapid breeding (generation time about 10 days at 25℃), innocuous, and low-cost maintenance. Also, *Drosophila* has a small genome of ~144Mbp which consists of about 17,738 genes and about 60% of these genes are conserved between *Drosophila* and humans. The availability of excellent genetic tools including balancer chromosomes, wide collection of mutants makes it a suitable animal model for various studies. In addition to this, with the help of reverse genetic tools such as clustered regularly interspaced palindromic repeats (CRISPR) it is now easy to perform molecular manipulations of *D. melanogaster* genome which further facilitates comparisons between it and other related organisms to study gene functions.

**The CRISPR/CAS9 system**

The **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats (CRISPR)/CRISPR Associated system (Cas) was first discovered in bacteria as a prokaryotic immune system that confers resistance against foreign DNA, either viral or plasmid and provides a form of acquired immunity (Ishino et al., 1987). The CRISPR/Cas system targets foreign DNA with a short complementary single-stranded RNA (CRISPR RNA (crRNA)) together with a sequence complementary to the palindromic repeat (trans-activating crRNA (tracrRNA)) that localizes the Cas9 nuclease to the target DNA sequence where the DNA target also does not need to be unique and can appear in multiple locations, all of which will be targeted by the Cas9 nuclease for cleavage. The crRNA binds on either strand of DNA depending on the complementarity with the target

sequence and targets Cas9 to cleave both strands (double strand break, DSB) which results in the silencing of that DNA sequence (Fig. 4).

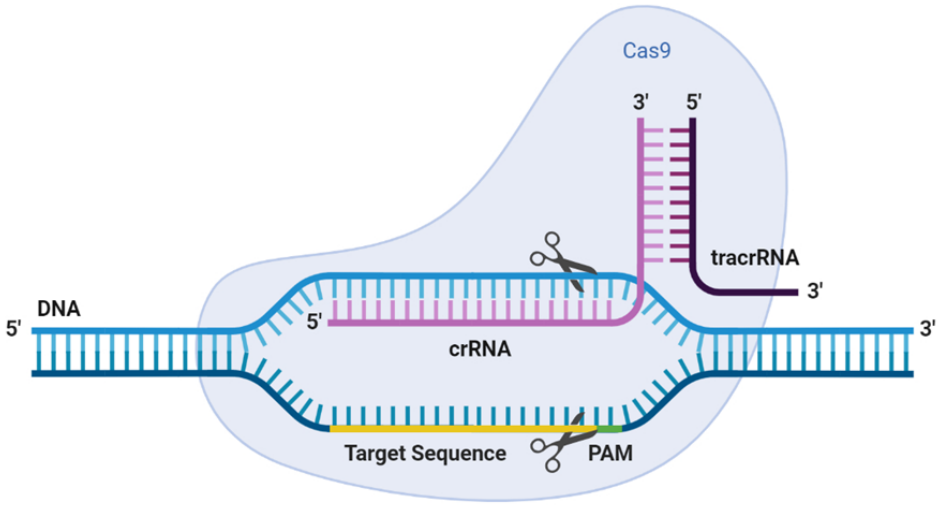


**Figure 4. A general overview of CRISPR/Cas9 system.** Cas9 nuclease forms a complex with crRNA and tracrRNA. Together this complex bind to the genomic DNA with the help of guide RNA (5' part of crRNA having complementarity with the target sequence) and after recognition of PAM sequence Cas9 cleaves the genomic DNA. Target specificity is determined by 20 nucleotides at the 5′ end of the crRNA, allowing the researcher to program the Cas9 cleavage.

Despite of its early discovery in the *Escherichia coli* genome in 1987 (Ishino et al., 1987), the function of CRISPR as a mechanism to safeguard against bacteriophages was not explored until 2007 where a study experimentally proved that CRISPR as an adaptive immune system (Barrangou et al., 2007). Later in 2010, a study on *Streptococcus thermophilus* showed that CRISPR/Cas makes a double strand break on phage and plasmid DNA (Garneau et al., 2010). From that point several

groups started exploring its role as an editing tool and started looking for simplified versions of this system. Soon after, a group led by Jennifer Doudna and Emmanuelle Charpentier started working on CRISPR system from *Streptococcus pyogenes* which has Cas9 protein where they were successful in developing and re-engineering Cas9 endonuclease into a simple and programmable two-component system by fusing crRNA and tracrRNA into a single-guide RNA which together with Cas9, could target the DNA specified by the guide RNA. Thereby allowing sequence specific targeting to nearly any sequence (depending on protospacer adjacent motif (PAM); 3-4 nucleotides downstream from the cut site and required for Cas nuclease) by manipulating the nucleotide sequence of the guide RNA (Jinek et al., 2012). It was further successfully utilized for genome editing for the very first time in human cell lines by a group led by Feng Zhang (Hsu et al., 2014). From there, it has since been successfully used in a variety of organisms including *D. melanogaster* (Bassett & Liu, 2014; Gratz et al., 2015; Kondo & Ueda, 2013; Port et al., 2014).

**Aim of the thesis**

Taken together, temperature compensation is a critical and fundamental feature of the circadian clocks. Role of the circadian clock gene *timeless* in the temperature compensation was suggested and temperature compensation mutants were identified in this gene. However, there is no information on the structure of TIM protein, despite the reasonable effort that well-established groups spent on their attempts to crystalize TIM. Therefore, the thesis aims at creating new mutants and to explore the role of *tim* in the temperature compensation using powerful genetic tools available in *Drosophila melanogaster*.

## References


Allada, R., White, N. E., So, W. V., Hall, J. C., & Rosbash, M. (1998). A mutant *Drosophila* homolog of mammalian clock disrupts circadian rhythms and transcription of *period* and *timeless*. *Cell*, 93(5), 791–804. https://doi.org/10.1016/S0092-8674(00)81440-3

Barion, A., & Zee, P. C. (2007). A clinical approach to circadian rhythm sleep disorders. *Sleep Medicine*, 8(6):566-77. https://doi.org/10.1016/j.sleep.2006.11.017

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., … Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, 315(5819), 1709-12. https://doi.org/10.1126/science.1138140

Bassett, A. R., & Liu, J.-L. (2014). CRISPR/Cas9 and genome editing in *Drosophila*. *Journal of Genetics and Genomics*, 41(1), 7–19. https://doi.org/http://dx.doi.org/10.1016/j.jgg.2013.12.004

Blau, J., & Young, M. W. (1999). Cycling *vrille* expression is required for a functional *Drosophila* clock. *Cell*, 99(6), 661-671. https://doi.org/10.1016/S0092-8674(00)81554-8

Boothroyd, C. E., Wijnen, H., Naef, F., Saez, L., & Young, M. W. (2007). Integration of light and temperature in the regulation of circadian gene expression in *Drosophila*. *PLoS Genetics*, *3*(4), e54. https://doi.org/10.1371/journal.pgen.0030054

Braunwald, E. (2012). On circadian variation of myocardial reperfusion injury. *Circulation Research*, 110(1), 6-7.

https://doi.org/10.1161/CIRCRESAHA.111.260265

Chatterjee, A., Tanoue, S., Houl, J. H., & Hardin, P. E. (2010). Regulation of gustatory physiology and appetitive behavior by the *Drosophila* circadian clock. *Current Biology*, 20(4), 300-309. https://doi.org/10.1016/j.cub.2009.12.055

Chiu, J. C., Low, K. H., Pike, D. H., Yildirim, E., & Edery, I. (2010). Assaying locomotor activity to study circadian rhythms and sleep parameters in *Drosophila. Journal of Visualized Experiments*, (43), e2157. https://doi.org/10.3791/2157

Collins, B. H., Rosato, E., & Kyriacou, C. P. (2004). Seasonal behavior in *Drosophila* melanogaster requires the photoreceptors, the circadian clock, and phospholipase C. *Proceedings of the National Academy of Sciences of the United States of America*, 101(7), 1945–1950. https://doi.org/10.1073/pnas.0308240100

Cyran, S. A., Buchsbaum, A. M., Reddy, K. L., Lin, M. C., Glossop, N. R. J., Hardin, P. E., … Blau, J. (2003). vrille, Pdp1, and dClock form a second feedback loop in the *Drosophila* circadian clock. *Cell*, 112(3), 329-341. https://doi.org/10.1016/S0092-8674(03)00074-6

Czeisler, C. A., Duffy, J. F., Shanahan, T. L., Brown, E. N., Mitchell, J. F., Rimmer, D. W., … Kronauer, R. E. (1999). Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science*, 284(5423), 2177-2181. https://doi.org/10.1126/science.284.5423.2177

Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D. L., … Kay, S. A. (1998). Closing the

circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. *Science*, 280(5369), 1599-1603. https://doi.org/10.1126/science.280.5369.1599

DeCoursey, P. J., Krulas, J. R., Mele, G., & Holley, D. C. (1997). Circadian performance of suprachiasmatic nuclei (SCN)-lesioned antelope ground squirrels in a desert enclosure. *Physiology and Behavior*, 62(5), 1099-1108. https://doi.org/10.1016/S0031-9384(97)00263-1

Emery, P. (1998). CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell*, 95(5), 669-679. https://doi.org/10.1016/S0092-8674(00)81637-2

Erren, T. C., & Reiter, R. J. (2008). A generalized theory of carcinogenesis due to chronodisruption. *Neuroendocrinology Letters*, 29(6), 815-821.

Ewer, J., Hamblen-Coyle, M., Rosbash, M., & Hall, J. C. (1990). Requirement for *period* gene expression in the adult and not during development for locomotor activity rhythms of imaginal *Drosophila melanogaster*. *Journal of Neurogenetics*, 7(1), 31-73. https://doi.org/10.3109/01677069009084151

Fujii, S., & Amrein, H. (2010). Ventral lateral and DN1 clock neurons mediate distinct properties of male sex drive rhythm in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 107(23), 10590-10595. https://doi.org/10.1073/pnas.0912457107

Fujii, S., Krishnan, P., Hardin, P., & Amrein, H. (2007). Nocturnal male sex drive in *Drosophila*. *Current Biology*, 17(3), 244-251. https://doi.org/10.1016/j.cub.2006.11.049

Garneau, J. E., Dupuis, M. È., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., … Moineau, S. (2010). The CRISPR/cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, 468, 67-71. https://doi.org/10.1038/nature09523

Gekakis, N., Saez, L., Delahaye-Brown, A.-M., Myers, M. P., Sehgal, A., Young, M. W., & Weitz, C. J. (1995). Isolation of *timeless* by PER protein interaction: defective interaction between *timeless* protein and long-period mutant PER^L. *Science*, 270(5237), 811–815. https://doi.org/10.1126/science.270.5237.811

Glaser, F. T., & Stanewsky, R. (2005). Temperature synchronization of the *Drosophila* circadian clock. *Current Biology*, 15(15), 1352–1363. https://doi.org/10.1016/j.cub.2005.06.056

Glossop, N. R. J., Houl, J. H., Zheng, H., Ng, F. S., Dudek, S. M., & Hardin, P. E. (2003). VRILLE feeds back to control circadian transcription of clock in the *Drosophila* circadian oscillator. *Neuron*, 37(2), 249-261. https://doi.org/10.1016/S0896-6273(03)00002-3

Goto, S. G., & Denlinger, D. L. (2002). Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: *period*, *timeless*, *cycle* and *cryptochrome*. *Journal of Insect Physiology*, 48(8), 803–816. https://doi.org/10.1016/S0022-1910(02)00108-7

Gratz, S. J., Rubinstein, C. D., Harrison, M. M., Wildonger, J., &

O'Connor-Giles, K. M. (2015). CRISPR-Cas9 genome editing in *Drosophila*. *Current Protocols in Molecular Biology*, 111(1), 31.2.1-31.2.20. https://doi.org/10.1002/0471142727.mb3102s111

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

Hamblen, M. J., White, N. E., Emery, P. T. J., Kaiser, K., & Hall, J. C. (1998). Molecular and behavioral analysis of four period mutants in *Drosophila melanogaster* encompassing extreme short, novel long, and unorthodox arrhythmic types. *Genetics*, 149(1), 165-178.

Hastings, J. W., & Sweeney, B. M. (1957). On the mechanism of temperature independence in a biological clock. *Proceedings of the National Academy of Sciences*, 43(9), 804-811. https://doi.org/10.1073/pnas.43.9.804

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, 157(6), 1262-1278. https://doi.org/10.1016/j.cell.2014.05.010

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., & Nakatura, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isoenzyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of Bacteriology*, 169(12), 5429-5433. https://doi.org/10.1128/jb.169.12.5429-5433.1987

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA – Guided DNA

endonuclease in adaptive bacterial immunity. *Science (New York, N.Y.)*, 337(6096), 816–821. https://doi.org/10.1126/science.1225829

Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., & Rosbash, M. (2007). *Clockwork Orange* is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. *Genes and Development*, 21, 1675-1686. https://doi.org/10.1101/gad.1552607

Kloss, B., Price, J. L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C. S., & Young, M. W. (1998). The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. *Cell*, 94(1), 97–107. https://doi.org/10.1016/S0092-8674(00)81225-8

Ko, H. W., Jiang, J., & Edery, I. (2002). Role for *Slimb* in the degradation of *Drosophila Period* protein phosphorylated by *Doubletime*. *Nature*, 420, 673-678. https://doi.org/10.1038/nature01272

Kondo, S., & Ueda, R. (2013). Highly Improved gene targeting by germline-specific Cas9 expression in Drosophila. *Genetics*, 195(3), 715–721. https://doi.org/10.1534/genetics.113.156737

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

Konopka, R. J., Hamblen-Coyle, M. J., Jamison, C. F., & Hall, J. C. (1994). An ultrashort clock mutation at the *period* locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. *Journal of Biological Rhythms*, 9(3-4), 189-216. https://doi.org/10.1177/074873049400900303

Konopka, R. J., Pittendrigh, C., & Orr, D. (1989). Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. *Journal of Neurogenetics*, 6(1), 1-10. https://doi.org/10.3109/01677068909107096

Krishnan, B., Dryer, S. E., & Hardin, P. E. (1999). Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature*, 400, 375-378. https://doi.org/10.1038/22566

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

Lévi, F. (2000). Therapeutic implications of circadian rhythms in cancer patients. *Novartis Foundation Symposium*, 227. https://doi.org/10.1002/0470870796.ch8

Lewy, A. J., Lefler, B. J., Emens, J. S., & Bauer, V. K. (2006). The circadian basis of winter depression. *Proceedings of the National Academy of Sciences of the United States of America*, 103(19), 7414-7419. https://doi.org/10.1073/pnas.0602425103

Lim, C., Chung, B. Y., Pitman, J. L., McGill, J. J., Pradhan, S., Lee, J., … Allada, R. (2007). clockwork orange Encodes a Transcriptional Repressor Important for Circadian-Clock Amplitude in *Drosophila*. *Current Biology*, 17(12), 1082-1089. https://doi.org/10.1016/j.cub.2007.05.039

Lin, J.-M., Kilman, V. L., Keegan, K., Paddock, B., Emery-Le, M.,

Rosbash, M., & Allada, R. (2002). A role for casein kinase 2alpha in the *Drosophila* circadian clock. *Nature*, 420(6917), 816–820. https://doi.org/10.1038/nature01235

Liu, Y. (1998). How temperature changes reset a circadian oscillator. *Science*, 281(5378), 825–829. https://doi.org/10.1126/science.281.5378.825

Lyons, L. C., & Roman, G. (2009). Circadian modulation of short-term memory in drosophiia. *Learning and Memory*, 16, 19-27. https://doi.org/10.1101/lm.1146009

Majercak, J., Sidote, D., Hardin, P. E., & Edery, I. (1999). How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron*, 24(1), 219–230. https://doi.org/10.1016/S0896-6273(00)80834-X

Manjunatha, T., Dass, S. H., & Sharma, V. K. (2008). Egg-laying rhythm in *Drosophila melanogaster*. *Journal of Genetics*, 87(5), 495–504. https://doi.org/10.1007/s12041-008-0072-9

Martin Anduaga, A., Evantal, N., Patop, I. L., Bartok, O., Weiss, R., & Kadener, S. (2019). Thermosensitive alternative splicing senses and mediates temperature adaptation in *Drosophila*. *ELife*, 8, e44642. https://doi.org/10.7554/eLife.44642

Martinek, S., Inonog, S., Manoukian, A. S., & Young, M. W. (2001). A role for the segment polarity gene *shaggy*/GSK-3 in the *Drosophila* circadian clock. *Cell*, 105(6), 769–779. https://doi.org/10.1016/S0092-8674(01)00383-X

Matsumoto, A, Tomioka, K., Chiba, Y., & Tanimura, T. (1999). *tim*$^{rit}$ lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. *Molecular and Cellular Biology*, 19(6), 4343–4354. https://doi.org/10.1128/MCB.19.6.4343

Matsumoto, Akira, Ukai-Tadenuma, M., Yamada, R. G., Houl, J., Uno, K. D., Kasukawa, T., … Ueda, H. R. (2007). A functional genomics strategy reveals *clockwork orange* as a transcriptional regulator in the *Drosophila* circadian clock. *Genes and Development*, 21, 1687-1700. https://doi.org/10.1101/gad.1552207

McDonald, M. J., Rosbash, M., & Emery, P. (2001). Wild-type circadian rhythmicity is dependent on closely spaced E boxes in the *Drosophila timeless* promoter. *Molecular and Cellular Biology*, 21(4), 1207-1217. https://doi.org/10.1128/mcb.21.4.1207-1217.2001

Menet, J. S., Abruzzi, K. C., Desrochers, J., Rodriguez, J., & Rosbash, M. (2010). Dynamic PER repression mechanisms in the *Drosophila* circadian clock: From on-DNA to off-DNA. *Genes and Development*, 24, 358-367. https://doi.org/10.1101/gad.1883910

Mwimba, M., Karapetyan, S., Liu, L., Marqués, J., McGinnis, E. M., Buchler, N. E., & Dong, X. (2018). Daily humidity oscillation regulates the circadian clock to influence plant physiology. *Nature Communications*, 9, 4290. https://doi.org/10.1038/s41467-018-06692-2

Myers, M P, Rothenfluh, A., Chang, M., & Young, M. W. (1997). Comparison of chromosomal DNA composing *timeless* in *Drosophila*

*melanogaster* and *D. virilis* suggests a new conserved structure for the TIMELESS protein. *Nucleic Acids Res*, 25(23), 4710–4714. https://doi.org/10.1093/nar/25.23.4710

Myers, Michael P., Wager-Smith, K., Wesley, C. S., Young, M. W., & Sehgal, A. (1995). Positional cloning and sequence analysis of the *Drosophila* clock gene, *timeless*. *Science*, 270(5237), 805–808. https://doi.org/10.1126/science.270.5237.805

Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S., & Johnson, C. H. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 95(15), 8660-8664. https://doi.org/10.1073/pnas.95.15.8660

Peschel, N., Chen, K. F., Szabo, G., & Stanewsky, R. (2009). Light-dependent interactions between the *Drosophila* circadian clock factors *cryptochrome*, *jetlag*, and *timeless*. *Current Biology*, 19(3), 241–247. https://doi.org/10.1016/j.cub.2008.12.042

Pittendrigh, C. S. (1954). On temperature independence in the clock system controlling emergence time in *Drosophila*. *Proceedings of the National Academy of Sciences*, 40(10), 1018-1029. https://doi.org/10.1073/pnas.40.10.1018

Pittendrigh, C. S. (1967). Circadian systems. I. The driving oscillation and its assay in *Drosophila pseudoobscura*. *Proceedings of the National Academy of Sciences of the United States of America*, 58(4), 1762-1767. https://doi.org/10.1073/pnas.58.4.1762

Pittendrigh, C. S. (1993). Temporal organization: Reflections of a

darwinian clock-watcher. *Annual Review of Physiology*, 55, 17-54. https://doi.org/10.1146/annurev.ph.55.030193.000313

Port, F., Chen, H.-M., Lee, T., & Bullock, S. L. (2014). Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*. *Proceedings of the National Academy of Sciences*, 111(29), E2967–E2976. https://doi.org/10.1073/pnas.1405500111

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

Richier, B., Michard-Vanhée, C., Lamouroux, A., Papin, C., & Rouyer, F. (2008). The *clockwork orange Drosophila* protein functions as both an activator and a repressor of clock gene expression. *Journal of Biological Rhythms*, 23(2), 103–116. https://doi.org/10.1177/0748730407313817

Rosato, E., & Kyriacou, C. P. (2006). Analysis of locomotor activity rhythms in Drosophila. *Nature Protocols*, 1, 559–568. https://doi.org/10.1038/nprot.2006.79

Rothenfluh, A., Young, M. W., & Saez, L. (2000). A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. *Neuron*, 26(2), 505–514. https://doi.org/10.1016/S0896-6273(00)81182-4

Rutila, J. E., Suri, V., Le, M., So, W. V., Rosbash, M., & Hall, J. C. (1998). CYCLE is a second bHLH-PAS clock protein essential for

circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell*, 93(5), 805-814. https://doi.org/10.1016/S0092-8674(00)81441-5

Rutila, J. E., Zeng, H., Le, M., Curtin, K. D., Hall, J. C., & Rosbash, M. (1996). The *tim*(SL) mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. *Neuron*, 17(5), 921–929. https://doi.org/10.1016/S0896-6273(00)80223-8

Sathyanarayanan, S., Zheng, X., Xiao, R., & Sehgal, A. (2004). Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell*, 116(4), 603-615. https://doi.org/10.1016/S0092-8674(04)00128-X

Scheer, F. A. J. L., Hilton, M. F., Mantzoros, C. S., & Shea, S. A. (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proceedings of the National Academy of Sciences of the United States of America*, 106(11), 4453-4458. https://doi.org/10.1073/pnas.0808180106

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

Shakhmantsir, I., Nayak, S., Grant, G. R., & Sehgal, A. (2018). Spliceosome factors target *timeless* (*tim*) mRNA to control clock protein accumulation and circadian behavior in *Drosophila*. *ELife*, 7, e39821. https://doi.org/10.7554/eLife.39821

Spiegel, K., Knutson, K., Leproult, R., Tasali, E., & Van Cauter, E. (2005). Sleep loss: A novel risk factor for insulin resistance and Type 2 diabetes. *Journal of Applied Physiology*, 99(5), 2008-2019. https://doi.org/10.1152/japplphysiol.00660.2005

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S. A., … Hall, J. C. (1998). The *cry*b mutation identifies *cryptochrome* as a circadian photoreceptor in *Drosophila*. *Cell*, 95(5), 681–692. https://doi.org/10.1016/S0092-8674(00)81638-4

Sun, Z. S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., & Lee, C. C. (1997). RIGUI, a putative mammalian ortholog of the *Drosophila period* gene. *Cell*, 90(6), 1003-1011. https://doi.org/10.1016/S0092-8674(00)80366-9

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M., & Sakaki, Y. (1997). Circadian oscillation of a mammalian homologue of the *Drosophila period* gene. *Nature*, 389, 512-516. https://doi.org/10.1038/39086

Vosshall, L., Price, J., Sehgal, A., Saez, L., & Young, M. (1994). Block in nuclear localization of *period* protein by a second clock mutation, *timeless*. *Science*, 263(5153), 1606–1609. https://doi.org/10.1126/science.8128247

Wijnen, H., Naef, F., Boothroyd, C., Claridge-Chang, A., & Young, M. W. (2006). Control of daily transcript oscillations in *Drosophila* by light and the circadian clock. *PLoS Genetics*, 2(3), e39. https://doi.org/10.1371/journal.pgen.0020039

Woelfle, M. A., Ouyang, Y., Phanvijhitsiri, K., & Johnson, C. H. (2004).

The adaptive value of circadian clocks: An experimental assessment in cyanobacteria. *Current Biology*, 14(16), 1481-1486. https://doi.org/10.1016/j.cub.2004.08.023

Wülbeck, C., Szabo, G., Shafer, O. T., Helfrich-Förster, C., & Stanewsky, R. (2005). The novel *Drosophila tim*$^{blind}$ mutation affects behavioral rhythms but not periodic eclosion. *Genetics*, 169(2), 751–766. https://doi.org/10.1534/genetics.104.036244

Yoshii, T., Heshiki, Y., Ibuki-Ishibashi, T., Matsumoto, A., Tanimura, T., & Tomioka, K. (2005). Temperature cycles drive *Drosophila* circadian oscillation in constant light that otherwise induces behavioural arrhythmicity. *European Journal of Neuroscience*, 22(5), 1176-1184. https://doi.org/10.1111/j.1460-9568.2005.04295.x

Yu, W., Zheng, H., Houl, J. H., Dauwalder, B., & Hardin, P. E. (2006). PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes and Development*, 20, 723-733. https://doi.org/10.1101/gad.1404406

Zylka, M. J., Shearman, L. P., Weaver, D. R., & Reppert, S. M. (1998). Three *period* homologs in mammals: Differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron*, 20(6), 1103-1110. https://doi.org/10.1016/S0896-6273(00)80492-4

# Chapter 1

New *Drosophila* circadian clock mutants affecting temperature compensation induced by targeted mutagenesis of *timeless*

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# New *Drosophila* Circadian Clock Mutants Affecting Temperature Compensation Induced by Targeted Mutagenesis of *Timeless*

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*Drosophila melanogaster* has served as an excellent genetic model to decipher the molecular basis of the circadian clock. Two key proteins, PERIOD (PER) and TIMELESS (TIM), are particularly well explored and a number of various arrhythmic, slow, and fast clock mutants have been identified in classical genetic screens. Interestingly, the free running period (tau, τ) is influenced by temperature in some of these mutants, whereas τ is temperature-independent in other mutant lines as in wild-type flies. This, so-called "temperature compensation" ability is compromised in the mutant *timeless* allele *"ritsu"* ($tim^{rit}$), and, as we show here, also in the $tim^{blind}$ allele, mapping to the same region of TIM. To test if this region of TIM is indeed important for temperature compensation, we generated a collection of new mutants and mapped functional protein domains involved in the regulation of τ and in general clock function. We developed a protocol for targeted mutagenesis of specific gene regions utilizing the CRISPR/Cas9 technology, followed by behavioral screening. In this pilot study, we identified 20 new *timeless* mutant alleles with various impairments of temperature compensation. Molecular characterization revealed that the mutations included short in-frame insertions, deletions, or substitutions of a few amino acids resulting from the non-homologous end joining repair process. Our protocol is a fast and cost-efficient systematic approach for functional analysis of protein-coding genes and promoter analysis *in vivo*. Interestingly, several mutations with a strong temperature compensation defect map to one specific region of TIM. Although the exact mechanism of how these mutations affect TIM function is as yet unknown, our *in silico* analysis suggests they affect a putative nuclear export signal (NES) and phosphorylation sites of TIM. Immunostaining for PER was performed on two TIM mutants that display longer τ at 25°C and complete arrhythmicity at 28°C. Consistently with the behavioral phenotype, PER immunoreactivity was reduced in circadian clock neurons of flies exposed to elevated temperatures.

**Keywords: circadian clock, reverse genetics, screening, candidate genes, temperature compensation, CRISPR-CAS9, *Drosophila melanogaster***

## INTRODUCTION

Circadian clocks orchestrate the physiology, metabolism, and behavior of living organisms to be optimally aligned to the periodic day and night changes in the environment. For that reason, circadian clocks "keep ticking" even under constant conditions with a free running period (*tau*, τ) close to 24 h. A crucial functional feature of circadian clocks is their ability to run with a comparable speed at a wide range of physiological temperatures, a phenomenon termed "temperature compensation." From a mechanistic point of view, these biological oscillators are a series of interconnected biochemical reactions, which involve transcriptional and translational feedback loops. The exceptional genetic tools available in the fruit fly, *Drosophila melanogaster*, have enabled the identification and detailed analysis of the functional components of the circadian system and their interactions. Many excellent and detailed reviews are available on this topic (Hardin, 2011; Ozkaya and Rosato, 2012; Tataroglu and Emery, 2015; Tomioka and Matsumoto, 2015).

At the core of the fruit fly's circadian clock, the transcription factors CLOCK (CLK) and CYCLE (CYC) drive the expression of genes with E-box motif(s) in the promoter region, including *period* (*per*) and *timeless* (*tim*). PER and TIM proteins slowly accumulate, dimerize in the cytoplasm, and later start to translocate to the cell nucleus, where they inhibit CLK–CYC mediated transcription (Darlington et al., 1998; Glossop et al., 1999). As a result of this negative feedback loop, *per* and *tim* mRNA is repressed, which consequently results in depletion of PER and TIM proteins, allowing the whole cycle to start again with a new round of CLK-CYC mediated transcription. Several kinases and phosphatases tightly regulate the stability of PER and TIM, fine-tuning the pace of the oscillator to roughly 24 h (Price et al., 1998; Martinek et al., 2001; Sathyanarayanan et al., 2004; Agrawal and Hardin, 2016). Additional interconnected transcription/translational feedback loops that contribute to the circadian system were described in *Drosophila* as well as other insects. The PER/TIM feedback loop model was established and further refined through a combination of immunocytochemistry (ICC) (Siwicki et al., 1988), time-course expression profiling (Hardin et al., 1990, 1992), protein biochemical approaches addressing phosphorylation (Edery et al., 1994; Chiu et al., 2011), glycosylation (Li et al., 2019), protein coexpression in *Drosophila* Schneider cell culture (Saez and Young, 1996; Nawathean and Rosbash, 2004; Meyer et al., 2006), and yeast two-hybrid experiments (Rutila et al., 1996). But the key starting point in the *per* and *tim* research was the identification of mutants in extensive genetic screens using either chemical mutagens (Konopka and Benzer, 1971; Konopka et al., 1994; Rothenfluh et al., 2000a), or P-element mobilization (Sehgal et al., 1994). Additionally, spontaneous clock mutations were recovered from wild populations (Matsumoto et al., 1999), or laboratory stocks (Hamblen et al., 1998). Importantly, not only null mutations were obtained, but also mutants with altered protein sequences resulting in faster or slower τ in both *per* (Konopka and Benzer, 1971; Konopka et al., 1994; Hamblen et al., 1998) and *tim* (Matsumoto et al., 1999; Rothenfluh et al., 2000a,b; Wülbeck et al., 2005) genes.

The protein–protein interaction between PER and TIM is a complex and dynamic event (Meyer et al., 2006), including PER homodimerization (Landskron et al., 2009), multiple sequential phosphorylations (Martinek et al., 2001; Ko et al., 2010; Chiu et al., 2011), dephosphorylations (Sathyanarayanan et al., 2004; Fang et al., 2007), and possibly additional posttranslational modifications (Li et al., 2019). A key feature of the negative feedback loop in *Drosophila* is the ∼ 6 h delay that exists between the cytoplasmic accumulation and nuclear translocation of PER and TIM. Both PER and TIM proteins contain a nuclear localization signal (NLS) and cytoplasmic localization domain (CLD) (Saez and Young, 1996). Transgenic flies with mutated TIM NLS have a slower τ, and even though PER and TIM reach high cytoplasmic levels, their nuclear translocation is substantially reduced (Saez et al., 2011). Nuclear entry of PER and TIM requires Importin α1 (IMPα1), which specifically interacts with TIM (Jang et al., 2015). TIM–IMPα1 interaction is abolished by $TIM^{PL}$ (proline 115 to leucine substitution) or $TIM^{TA}$ (threonine 113 to alanine) mutations. Consistently, $tim^{PL}$ and $tim^{TA}$ flies are arrhythmic and $TIM^{PL}$ remains cytoplasmic in circadian clock neurons (Hara et al., 2011). Additionally, TIM is actively exported from the nucleus by CRM1 and this export is affected by interaction with PER (Ashmore et al., 2003). Another mutation with slower τ and abnormal response to light pulses, $tim^{blind}$, encodes a protein with impaired nuclear accumulation. One of the amino acid substitutions in $TIM^{blind}$ is located within a putative nuclear export signal (NES) (Wülbeck et al., 2005). Collectively, these observations demonstrate the crucial importance of precise regulation of subcellular TIM localization.

Along with light, a primary cue for entrainment, *Drosophila* circadian clocks can be entrained by regular alternations of warmer and colder temperatures (Glaser and Stanewsky, 2005; Sehadova et al., 2009). Also, the distribution of daily activity differs between warm and cold days, which is regulated by temperature-dependent splicing of a *per* intron located within the 3′ untranslated region of mRNA in *D. melanogaster* (Majercak et al., 1999; Zhang et al., 2018). However, at constant conditions, the period length of the circadian clock remains unchanged over a wide range of physiological temperatures. Temperature compensation is a general feature of circadian clocks (Pittendrigh, 1954; Hastings and Sweeney, 1957) conserved from cyanobacteria to mammals (Izumo et al., 2003; Nakajima et al., 2005). In essence, any (bio)chemical reaction runs faster with rising temperature (Arrhenius, 1889), therefore, temperature compensation mechanism should involve multiple reactions, which are differently influenced by temperature, opposing each other (Ruoff, 1992). For example, in the red bread mold, *Neurospora crassa*, temperature-dependent alternative splicing of *frequency* results in long and short FREQUENCY protein isoforms, which have opposing effect on clock speed (Diernfellner et al., 2007). In mammals, distinct phosphorylation of PER2 is important for a temperature-compensated circadian clock (Zhou et al., 2015). Moreover, recently it was shown that the overall activity of the important PER2 kinase CK1δ is temperature-compensated, contributing to

temperature-independent τ in the mammals (Shinohara et al., 2017). Interestingly, the τ of some period-altering *Drosophila* mutations remains constant over a wide range of temperatures (the circadian clock is well temperature-compensated), whereas others have temperature-dependent phenotypes. In the case of $per^{L}$, higher temperature further slows down τ from 27.8 h at 17°C to 30.5 h at 25°C (Konopka et al., 1989). A similar and even more profound trend was identified in $tim^{rit}$ where τ is 25.5 h at 24°C and rises to 35 h at 30°C (Matsumoto et al., 1999). An opposite temperature compensation abnormality was reported for $per^{SLIH}$ (*Some Like It Hot*), a spontaneous mutation frequently found in various laboratory stocks (Hamblen et al., 1998). Interestingly, the $tim^{SL}$ (*Suppressor of* $per^{Long}$) mutation eliminates the temperature compensation defect of $per^{L}$, whereas $tim^{SL}$ has no circadian phenotype on its own (Rutila et al., 1996).

Given the length of PER (1218aa) and TIM (1421aa) proteins, however, even the existing remarkable collection of mutants has not been sufficient to uncover all the regions important for the circadian clock machinery and particularly for temperature compensation. Here we discovered that the previously isolated $tim^{blind}$ allele is defective in temperature compensation similar to the neighboring $tim^{ritsu}$ allele. To further explore the role of this and other regions of TIM in temperature compensation and clock function, we performed a targeted CRISPR/CAS9 screen, challenging eight different TIM protein regions and isolated ∼20 new mutants with a functional circadian clock, but altered τ. Our data revealed that manipulation of one region of TIM in particular, consistently produces temperature compensation defects. In addition, we developed a screening protocol that is an efficient alternative to classical mutagenesis approaches (Price, 2005) or rescue experiments with modified transgenes (Baylies et al., 1992; Landskron et al., 2009).

## MATERIALS AND METHODS

### Fly Strains and *per*, *cry*, *tim* Combinations

Mutant and wild-type alleles of *per* ($per^{wt}$, $per^{S}$, $per^{T}$, $per^{SLIH}$, $per^{L}$) (Konopka and Benzer, 1971; Hamblen et al., 1998), *tim* ($tim^{wt}$, $tim^{blind}$, $tim^{S1}$, $tim^{L1}$, $tim^{rit}$, $tim^{UL}$) (Matsumoto et al., 1999; Rothenfluh et al., 2000a,b, Wülbeck et al., 2005), and *cry* ($cry^{01,02,03}$, $cry^{b}$, $cry^{m}$, and $cry^{wt}$) (Stanewsky et al., 1998; Busza et al., 2004; Dolezelova et al., 2007) genes were combined by genetics crosses using balancer chromosomes and if necessary, the presence of a particular allele was confirmed by sequencing.

### Locomotor Activity Measurement and Analysis

#### Constant Temperature

Two- to four-days-old males were $CO_2$ anesthetized and transferred to 70 mm tubes containing 5% sucrose in agar and loaded into the DAM2 TriKinetics system (Waltham, MA, United States), entrained to light:dark (LD, 12:12) conditions for 5 days and released to constant darkness (DD) for additional 10–14 days. The last 3 days were omitted from the analyses but were used to determine fly survival. To determine τ during the first 10 days in DD, chi-square periodogram analysis was performed using ActogramJ (Schmid et al., 2011) and double-plotted actograms were eye inspected in parallel. The same temperature (17, 20, 25, 28°C) was used during the LD entrainment and DD conditions, with the exception of the temperature step-up protocol (see below). For the $tim^{blind}$, $tim^{A1128V}$, and $tim^{L1131M}$ mutations generated by site-directed mutagenesis and homologous recombination, flies were exposed to LD for 3 days, followed by 5–7 days in DD at constant temperatures of 18, 25, or 29°C. Period length and their significance (RS values) were determined using autocorrelation and Chi-square periodogram analysis functions of the fly tool box implemented in MATLAB (MathWorks) (Levine et al., 2002). Period values with associated RS values ≥ 1.5 were considered rhythmic (Levine et al., 2002).

#### Temperature Step-Up Protocol

To be able to measure τ in individual flies at different temperatures, 2- to 4-days-old males were $CO_2$ anesthetized, loaded into DAM2 TriKinetics system, and entrained in LD (12:12) regime at 20°C for 4 days and then released to DD for 7 days at 20°C (MIR 154 incubators, Panasonic). On eighth day, the temperature was raised to 28°C during 24 h (1°C every 3 h) and locomotor activity was recorded for additional 7 days at 28°C. Step-up protocol was used during the screen to facilitate identification of even subtle temperature-dependent change in τ and to enhance throughput during screening.

### Interspecific Comparison of TIM Proteins

Protein sequences of TIM representing dipteran flies *Chymomyza costata* (Kobelkova et al., 2010; Poupardin et al., 2015) and *Musca domestica* (Bazalova and Dolezel, 2017), lepidopteran moth *Ephestia kuehniella* (Kobelkova et al., 2015), heteropteran bug *Pyrrhocoris apterus* used frequently in research of photoperiodic clock (Pivarciova et al., 2016; Urbanova et al., 2016; Kotwica-Rolinska et al., 2017), and basal insect species German cockroach *Blatella germanica* (Bazalova et al., 2016) and firebrat *Thermobia domestica* (Kamae and Tomioka, 2012) were aligned in Geneious 11 using Clustal W algorithm.

### Nuclear Export Signal (NES) Prediction

The putative NESs were identified using motif search in Geneious software according to following consensus patterns after Fung et al. (2015) and Ashmore et al. (2003), respectively, where X corresponds to any amino acid, L = leucine, V = valine, I = isoleucine, F = phenylalanine, M = methionine, and square brackets indicate alternatives:

NES-1a (Fung et al.) [LVIFM]XXX[LVIFM]XX[LVIFM]X[LVIFM]
NES-1b (Fung et al.) [LVIFM]XX[LVIFM]XX[LVIFM]X[LVIFM]
NES-1c (Fung et al.) [LVIFM]XXX[LVIFM]XXX[LVIFM]X[LVIFM]
NES-1d (Fung et al.) [LVIFM]XX[LVIFM]XXX[LVIFM]X[LVIFM]

NES-2 (Fung et al.) [LVIFM]X[LVIFM]XX[LVIFM]X[LVIFM]
NES-3 (Fung et al.) [LVIFM]XX[LVIFM]XXX[LVIFM]XX[LVIFM]
NES-1a-R (Fung et al.) [LVIFM]X[LVIFM]XX[LVIFM]XXX[LVIFM]
NES-1b-R (Fung et al.) [LVIFM]X[LVIFM]XX[LVIFM]XX[LVIFM]
NES-1c-R (Fung et al.) [LVIFM]X[LVIFM]XXX[LVIFM]XXX[LVIFM]
NES-1d-R (Fung et al.) [LVIFM]X[LVIFM]XXX[LVIFM]XX[LVIFM]
NES (Ashmore et al.) $Lx_{(1-3)}Lx_{(1-3)}Lx$[LVIM].

## Prediction of Phosphorylation Sites

The putative phosphorylation sites were predicted *in silico* using NetPhos 3.1 server at http://www.cbs.dtu.dk/services/NetPhos/ and scores higher than 0.5 were plotted in alignments.

## Gene Editing Inducing Non-homologous-End-Joining (NHEJ)—gRNA Design

Target sites were identified using CRISPR target finder[1] and gRNA design was validated according to parameters mentioned in Ren et al. (2014). To start transcription from U6 promoter 5′ guanine is required; therefore, target sites that lack this feature were extended by single guanine in the 5′ direction (see **Table 1** for gRNA sequences and their position on *tim*/TIM). To construct a gRNA expression vector with U6 promoter upstream of *tim*-specific gRNA, two complementary 24-bp oligonucleotides (custom synthesized, Generi Biotech Ltd.) were annealed to obtain a double-strand DNA with 4-bp overhangs compatible to BbsI-linearized pBFv-U6.2 vector (Kondo and Ueda, 2013) obtained from fly stocks of National Institute of Genetics, Japan (NIG-FLY). Plasmid and inserts were ligated with T4 DNA ligase overnight at 4°C and transformed to DH5α competent cells. Presence of the insert was confirmed by PCR and positive clones were sequenced.

## Gene Editing Inducing Homology Directed Repair (HDR)—gRNA Design

Target gRNA sites were selected so that Cas9 mediated cleavage was directed to a target locus of 100 bp upstream and downstream of the $tim^{blind}$ mutation. To avoid off target cleavage optimal target sites were identified using CRISPR target finder (see footnote 1). Two gRNA targets were chosen that are close to the target locus. Complementary target site oligos also contained a 5′ guanine for transcription from the U6 promoter and a 3 bp overhang compatible to BbsI sites. Oligos were annealed using standard primer annealing reactions and cloned into BbsI linearized pCFD3 plasmid (Port et al., 2014) via T4 DNA ligation.

[1]https://flycrispr.org/target-finder/

## Gene Editing Inducing HDR—Donor Plasmid Construction

Donor plasmids that contain the desired *tim* point mutations and all elements necessary for homologous recombination were constructed in three subsequent cloning steps. In each round of cloning the 1.5 kb 5′ homology arm and the 1.5 kb 3′ homology arm were individually PCR amplified using outside primers *tim*BMHRF and *tim*BMHRR2 in combination with respective internal primers. Outside primers *tim*BMHRF and *tim*BMHRR2 contain a 15 bp overhang for In-Fusion cloning that is homolog to linearized vector ends. Inside primers have 5′ 15–20 bp extensions that are complementary to each other in addition to one defined mutation for each round of cloning. In the initial round of cloning, Pam site mutations were introduced to avoid unwanted Cas9 cleavage within the donor plasmid. The two fragments (5′ homology arm and 3′ homology arm) were amplified from *y w* flies and assembled into plasmid pBS-KS-attB1-2-PT-SA-SD-0-2xTY1-V5 (Addgene) that was linearized with XbaI and HindIII using In-Fusion cloning. In a second round of cloning, the homology arms were amplified again using the pBS donor plasmid from the previous round as a template. Outside primers were as described above while the inside primers introduced a silent SalI site that can be used to screen for transformants. In-fusion cloning was used to assemble the fragments as described above. The resulting plasmid was then used in a final round of PCR to introduce the individual $tim^{blind}$ mutations A1128V, L1131M and for remaking the original $tim^{blind}$ double mutation (see **Table 2** for a detailed list of all primers).

## Transgenesis for NHEJ Mutagenesis

gRNA-encoding plasmids were purified with Qiagen miniprep kit and DNA was eluted in $H_2O$. Plasmids were diluted to concentration 100 ng/μl and injected into freshly laid embryos of $y^2$ $cho^2$ $v^1$ *P{nos-phiC31\int.NLS}X; attP2 (III)* stock (NIG-FLY#: TBX-0003) with embryonically expressed phiC31 integrase from transgene located on the X chromosome, attP landing site on the third chromosome, and *chocolate* and *vermillion* ($cho^2$ $v^1$) mutations on the X chromosome. G0 flies were crossed to $y^2$ $cho^2$ $v^1$*; Pr Dr/TM6C, Sb Tb* (NIG-FLY#: TBX-0010) and F1 offspring were selected for eye color rescue ($v^+$ transgene in the $cho^2$ $v^1$ background turns the eye color from light orange to dark brown). Strains with gRNA-encoding transgene were balanced with TM6C, *Sb Tb* and kept as stock.

## Transgenesis for HDR Experiments

Donor plasmids containing the desired mutation along with gRNA plasmids were verified by sequence analysis and scaled up for injections using Qiagen plasmid midiprep; 6 μg of each plasmid was precipitated and eluted in injection buffer. gRNA construct and donor plasmids were mixed prior to injection and the mix was injected into freshly laid embryos of *nos*-Cas9 flies (Port et al., 2014). Surviving adults were backcrossed in batch crosses to *y w*, *Bl/CyO*, + flies to balance second chromosome modifications

**TABLE 1 |** List of gRNA used in this study.

| Region in TIM | gRNA name | gRNA sequence[PAM] (5′→3′) | Protein sequence corresponding to gRNA |
|---|---|---|---|
| upstream of UL (two gRNAs) | UL rev | ACAGAGAGGCTTCGAACCAG[AGG] | 268-SLWFEASLS-276 |
| | UL fw | TAATACCTCGCCCCCCAAAC[AAG] | 284-SNTSPPKQ-291 |
| NES$^{776-785}$ | fw 781-789 | CCTACTCATTCTGGACAGTT[CGG] | 781-LLLILDSSA-789 |
| NES$^{1015-1023}$ | fw 1017-1025 | CCTGCTGGACCTGATCATTA[AGG] | 1017-ILLDLIIKE-1025 |
| NES$^{1093-1104}$ | rev 1090-1098 | GGAGCAGGAGAACAAAAGGC[TGG] | 1090-YQPFVLLLH-1098 |
| ritsu and blind (two gRNAs) | rev ritsu | GTCTCCGGTGTCCAGTAGTC[CGG] | 1116-PDYWTPET-1123 |
| | fw blind | GTACGGACTCGCCAAAAAGC[TGG] | 1124-MYGLAKKLG-1132 |
| NES$^{1166-1174}$ | rev 1171-1179 | TATCGCCGAGATCCACGTCC[AGG] | 1171-SLDVDLGDT-1179 |
| Unspliced$^{1387}$ (EKEKEL$^{Kstop}$) | fw unspliced 1387 | AAAGTGAGTGCGATTGAGCC[TGG] | Mostly intronic sequence |

*First column indicates protein region of interest. The name of gRNA specifies its directionality (fw—forward, rev—revers) and position corresponding to protein sequence. Square brackets after each gRNA indicate protospacer adjacent motif (PAM) sequence. Gray background highlights situation when construct contains two gRNAs. Superscript indicates protein sequence resulting from retained intron.*

**TABLE 2 |** Primers used for HDR experiments.

| Primer name | Sequence | Used for |
|---|---|---|
| timBMHRF | GATGGTCGACTCTAGACGGAGAGTTTGTCAATGACTGC | Outside primer for amplifying 5′ homology region |
| timBMHRR2 | GATGGTCGACAAGCTTCTTGAGACGTAGACGGAGTCGG | Outside primer for amplifying 3′ homology region |
| TimBMPAMmutF1a | GGAGACAATGTATGGACTCGCCAAAAAGCTGGGAC | Introduces Pam site mutation for gRNA target 1 |
| TimBMPAMmutR1a | AGTCCATACATTGTCTCCGGTGTCCAGTAGT | Introduces Pam site mutation for gRNA target 1 |
| timSalIF | CGTGAGTTAAAGTCGACCACAGAAAAAAACAACCCATTTG | Introduces Sall site |
| timSalIR | GGTCGACTTTAACTCACGTTTGTCCAGC | Introduces Sall site |
| timA1128VF | CAATGTATGGACTCGTCAAAAAGCTGGGACCGCT | Introduces A1128V mutation |
| timA1128VR | GACGAGTCCATACATTGTCTCCGGTGTCCA | Introduces A1128V mutation |
| timL1131MF | GACTCGTCAAAAAGATGGGACCGCTGGACAAACG | Introduces L1131M mutation for double *tim$^{blind}$* mutant |
| timL1131MR | CATCTTTTTGACGAGTCCATACATTGTCTCCGG | Introduces L1131M mutation for double *tim$^{blind}$* mutant |
| timL1131MFa | GACTCGCCAAAAAGATGGGACCGCTGGACAAACG | Introduces L1131M mutation |
| timL1131MRa | CATCTTTTTGGCGAGTCCATACATTGTCTCCGG | Introduces L1131M mutation |
| TimBMPAMmutF2a | GACTACTGGACACCAGAAACAATGTATGGACTCGCCA | Introduces Pam site mutation for gRNA target 2 |
| TimBMPAMmutF2b | GACTACTGGACACCAGAAACAATGTATGGACTCGTCA | Introduces Pam site mutation for gRNA target 2 |
| TimBMPAMmutR2a | TTCTGGTGTCCAGTAGTCCGGAATTCTGGCG | Introduces Pam site mutation for gRNA target 2 |
| ScreenTimF1 | CTCCCACTTCCGCAACAACAGAGTCTG | Molecular screen of transformants |
| ScreenTimR2 | GCTGCTTACCGAGCTGAGCGAGTTGCG | Molecular screen of transformants |
| timgRNAT1F | GTCGTGGACACCGGAGACAATGTA | gRNAtarget 1 |
| timgRNAT1R | AAACTACATTGTCTCCGGTGTCCAC | gRNAtarget 1 |
| timgRNAT2F | GTCGCGAGTCCGTACATTGTCTC | gRNAtarget 2 |
| timgRNAT2R | AAACGAGACAATGTACGGACTCGC | gRNAtarget 2 |

with CyO. Individual male and female flies from this cross were crossed again to *y w*, *Bl/CyO*, +. After egg deposition for 3–5 days, adult transformant flies were used for molecular screening.

Because initial attempts to introduce the A1128V mutation did not result in any positive transformants, a slightly different approach was used. The original *tim$^{blind}$* EMS stock was crossed to nosCas9 flies and embryos of this cross were injected with donor plasmids containing the A1128V mutation and the wild-type residue at position 1131 to back mutate the M at this position to L.

## Genetic Crosses Inducing NHEJ

The CAS9 editing procedure utilized fly strains and tools established by Kondo and Ueda (2013). Flies expressing Cas9 specifically in germ cells (nos-Cas9) from third chromosome insertion (NIG-FLY#: CAS-0003; *y$^2$ cho$^2$ v$^1$*; *P{nos-Cas9, y+, v+}3A/TM6C, Sb Tb*) were crossed with individual U6gRNA-encoded transgenic strains (also located on the third chromosome). Resulting offspring thus expressed both gRNA and CAS9 on third chromosome, which potentially targeted *tim* gene located on the second chromosome and induce insertions and deletions as a result of non-homologous-end-joining (NHEJ) mechanism. The resulting offspring were crossed to *y$^2$ cho$^2$ v$^1$*; *Sco/CyO* (NIG-FLY#: TBX-0007) to balance modified second chromosome by *CyO*. Males and females with second chromosome balancer were individually crossed again to *y$^2$ cho$^2$ v$^1$*; *Sco/CyO* flies to establish lines with identically modified second chromosomes (see **Supplementary Figure S1** for the crossing scheme).

## Behavioral Screening in NHEJ Experiments

To identify mutants, locomotor activity of eight males per line (homozygous for the second chromosome) was recorded in temperature step-up protocol and any alternations in locomotor activity pattern (arrhythmicity, change in τ) were identified from the double-plotted actograms. In parallel, reference strains with either functional ($w^{1118}$ or Canton S) or altered temperature compensation ($tim^{rit}$) were recorded as a negative and positive control. Phenotypes of putative mutant lines were further confirmed on a large sample in temperature step-up protocol and also independently at low (17°C), ambient (25°C), and high (28°C) constant temperatures.

## Molecular Screening in HDR Experiments

A total of 95 flies for each mutation were screened using PCR and restriction digests. In detail, a ∼800 bp target locus containing the expected mutations along with the introduced SalI restriction site was amplified by PCR using genomic DNA from individual flies; 1 μl of SalI was then added to half of the PCR and incubated for 2 h at 37°C. Resulting products were analyzed on agarose gels. The remaining PCR product of samples that showed digested products of the correct size was then used for sequencing to verify the presence of the desired mutations.

## Sequencing of Mutated Region

To characterize the molecular nature of the CRISPR/Cas9-induced mutations, target loci were first amplified by PCR using genomic DNA extracted from individual flies. One fly was crushed in 50 μl of Squishing buffer (10 mM Tris-HCl pH8; 1 mM EDTA; 25 mM NaCl; 200 g/ml Proteinase K), followed by incubation at 37°C for 30 min and later Proteinase K inactivation at 95°C for 3 min. Using 1 μl of the crude DNA extract as a template, a DNA sequence surrounding the target site was amplified by PCR for 35 cycles in a 10 μl reaction with 2X PPP Master Mix (Top-Bio, Prague, Czech Republic). The PCR products were analyzed by agarose gel electrophoresis and Sanger sequencing. Primers used for PCR and DNA sequencing are listed in **Table 3** and the actual number of obtained mutants is in **Table 4**.

## Immunocytochemistry

Seven-days-old males kept in LD regime and constant temperature of 17, 20, or 28°C were collected at specific time points (every 4 h) during the day; red light was used for collection in the dark time points. Flies were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; pH 7.4) for 1.5 h, washed in PBS twice, and brains were dissected. Next, tissues were fixed again for 45 min in 4% PFA and washed five times in PBS with an addition of 0.2% Triton X-100 (PBT). After that, brains were incubated in 5% normal goat serum (NGS) with an addition of 0.5% bovine serum albumin (BSA) for 30 min first at room temperature, and then incubated with primary anti-PER antibodies (rabbit, 1:5000, Stanewsky et al., 1997) for 3 days and with anti-PDF (mouse, 1:500, Hybridoma Bank) for 1 day at 4°C. Afterward, brains were washed six times in PBT/BSA and blocked in 5% NGS for 45 min. After that, goat anti-rabbit (conjugated with Cy3, 1:500, Jackson Immuno Research) and goat anti-mouse (conjugated with Alexa 488, 1:1000, Molecular Probes) secondary antibodies were applied overnight in 4°C. Finally, brains were washed twice in BSA, six times in PBT, and twice in PBS. Then, brains were mounted in Vectashield medium (Vector) and examined under a Zeiss Meta 510 laser scanning microscope. The identical laser settings were used for all images.

## Quantitative Comparison of Immunofluorescence Values

Collected images were analyzed using ImageJ software (NIH, Bethesda). PDF-immune-positive cells were marked (green channel) and PER fluorescence intensity (red channel) was measured in the selected area and outside of the PDF-expressing cells (background). Fluorescence intensity is represented by the mean gray value (the sum of the values of all pixels in the area divided by the number of pixels within the selection). The final value was obtained by subtracting the background value from the staining in the selected area. At least 10 brains per each time point were checked. Experiments were repeated three times.

## Compensation for Different Staining Affinity After Antibody Re-use

Brains from every genotype were isolated at ZT0 at 17, 25, and 28°C in parallel. After immunostaining, mean gray value was measured. The mean was used to calculate the ratio between the intensity staining in different temperatures for every genotype separately. Then the ratio between means at ZT0 at different temperatures from previous experiments was calculated, which allowed identifying the factor used for data compensation. This allowed us to avoid differences in staining intensity caused by changes in antibodies affinity after re-use.

## Statistical Analysis

The differences between τ were tested for statistical significance by one-way ANOVA with Tukey–Kramer's *post hoc* test using Graphpad7 (Prism) software. ICC staining intensities were compared by one-way ANOVA between genotypes for each timepoint, temperature, and cell type.

# RESULTS

## Genetic Interaction Between *tim*, *cry*, and *per*

We combined existing *cry* alleles ($cry^{01,02,03}$, $cry^{b}$, $cry^{m}$, and $cry^{wt}$) with available alleles of *per* ($per^{wt}$, $per^{S}$, $per^{T}$, $per^{SLIH}$, $per^{L}$) and *tim* ($tim^{wt}$, $tim^{blind}$, $tim^{S1}$, $tim^{L1}$, $tim^{rit}$, $tim^{UL}$). Locomotor activity was recorded in 65 genetic combinations at low (18°C), standard (25°C), and high (28°C) temperatures. In general, the observed free running periods were consistent with published data including previously reported temperature compensation defects of $per^{L}$ (Konopka et al., 1989), $tim^{rit}$ (Matsumoto et al., 1999), and $per^{SLIH}$ (Hamblen et al., 1998). Similarly, combination of $per^{L}$ with *cry* mutants ($cry^{01,02,03}$, $cry^{b}$, $cry^{m}$) resulted in flies

**TABLE 3 |** Primers used for amplification and subsequent sequencing of mutants.

| Targeted region | Note | Orientation | Primer sequence | Amplicon size (bp) |
|---|---|---|---|---|
| Upstream of UL | Used for sequencing | Forward | ACTCCTGTATCTGATGACC | 334 |
| | | Reverse | GATACTCCTGACCCTTGC | |
| | Used for detection of the in/del only | Forward | TACAAGGATCAGCATGTG | 185 |
| | | Reverse | GCCATTGCTGCCATTGT | |
| $NES^{776-785}$ | | Forward | GCGAAATGTCCGATCTGAGG | 188 |
| | | Reverse | CCCTACTGTGTTATGTGCTC | |
| $NES^{1015-1023}$ | | Forward | CCTCAGATGATGTTCAGGTG | 156 |
| | | Reverse | GCAGCACTCAATGAGGATCC | |
| $NES^{1093-1104}$ | | Forward | CCGGAAGGCGATCACATCAT | 217 |
| | | Reverse | GTCCGTACATTGTCTCCGGT | |
| ritsu and blind | Used for detection of the in/del only | Forward | GCAGTGGAACAACGAGCAAT | 225 |
| | | Reverse | TGTGGAATGACAAATGGGT | |
| | Used for detection of the in/del only | forward | CTCCACAAGCTGGGCATT | 132 |
| | | Reverse | CTTTAACTCACGTTTGTCCAGC | |
| | Used for sequencing | Forward | GATCACATCATGGAGCCGGTG | 565 |
| | | Reverse | TGAGCGAGTTGCGGGGTC | |
| $NES^{1166-1174}$ | | Forward | CCTCAAGTTCGACGCCAGTG | 238 |
| | | Reverse | GTTGCAGTGCTTCGTCTTGG | |
| $Unspliced^{1387}$ | | Forward | GGCTGGAAATGGATGTGGAC | 280 |
| | | Reverse | CTGTCAAACTGAGAGGTGAC | |

with temperature compensation defects comparable to $per^L$ alone (Fexová, 2010, M.Sc. thesis, and **Supplementary Table S1**), in agreement with the retraction note for Kaushik et al. (2007) (PLoS Biol 2016, 14:e1002403).

## $tim^{blind}$ Is a Temperature Compensation Mutant

$tim^{blind}$ was identified in a chemical mutagenesis screen for mutations altering *period* gene expression and contains two conservative amino acid substitutions, alanine to valine (V) at position 1128 and leucine (L) to methionine (M) at position 1131 (Wülbeck et al., 2005). While $tim^{blind}$ flies exhibit a long (26 h) free running period length of locomotor activity (measured at 25°C), the period length of eclosion rhythms (measured at 20°C) is normal (24.5 h) (Wülbeck et al., 2005). We therefore tested the possibility that $tim^{blind}$ mutants are defective in temperature compensation. Indeed, $tim^{blind}$ showed a normal τ of locomotor activity rhythms at 17°C (23.7 h), and gradually longer τ at higher temperatures (24.0 h at 20°C; 25.7 h at 25°C; 26.8 at 28°C; $P < 0.001$, **Figures 1B,D** and **Table 5**), confirming that this *tim* allele affects temperature compensation. In agreement with Wülbeck et al. (2005), the mutant is fully recessive (**Figures 1C,D**). One of the two $tim^{blind}$ amino-acid substitutions (L1131M) maps to one of the six potential NESs originally predicted for TIM (Ashmore et al., 2003), indicating that this mutation is responsible for the $tim^{blind}$ phenotypes (Wülbeck et al., 2005). To test this hypothesis, we introduced the individual substitutions as well as the double-mutant into the endogenous *tim* gene, using site-directed mutagenesis combined with CRISPR/Cas9 mediated homologous recombination (Port et al., 2014; see section "Materials and Methods"). Locomotor behavior of the resulting mutants ($tim^{A1128V}$, $tim^{L1131M}$, $tim^{blind-2.1}$) was analyzed in DD at 18, 25, and 29°C to determine rhythmicity and potential defects in temperature compensation. Surprisingly, none of the single mutants recapitulated the phenotypes of the original $tim^{blind}$ double-mutant. The $tim^{A1128V}$ mutation led to high percentage of arrhythmicity, ranging from 57% at 18°C to 77% at 29°C (**Figure 1E** and **Table 5**). Although the remaining rhythmic flies had variable periods at all three temperatures, overall they still indicate a potential period lengthening with increasing temperatures (**Figures 1E,H** and **Table 5**). The severe impact of the $tim^{A1128V}$ mutation on rhythmicity is surprising, given the conservative nature of this amino acid replacement. In contrast, the predicted NES mutation $tim^{L1131M}$ basically had no effect on rhythmicity and period length at any temperature. Between 84% (18°C) and 63% (29°C) of the mutant flies were rhythmic with period values slightly below 24 h at all temperatures (**Figures 1F,H** and **Table 5**). On the other hand, the re-engineered $tim^{blind}$ double-mutant ($tim^{blind-2.1}$) showed temperature-dependent period lengthening and robust rhythmicity at all temperatures, closely matching the phenotypes of the $tim^{blind}$ EMS allele (**Figures 1G,H** and **Table 5**, Wülbeck et al., 2005). At 25°C, the period length of $tim^{blind-2.1}$ is about 1 h shorter compared to $tim^{blind}$ (**Figures 1B,G** and **Table 5**, Wülbeck et al., 2005), while at 29°C $tim^{blind-2.1}$ it is about 1 h longer compared to $tim^{blind}$ at 28°C. Because the experiments with the original $tim^{blind}$ allele and $tim^{blind-2.1}$ were performed in different laboratories (České Budějovice and Münster, respectively), small differences in the experimental set-up (e.g., temperature), or period length determinations could explain these discrepancies. Importantly, both the synthetic and original $tim^{blind}$ alleles show a pronounced defect in

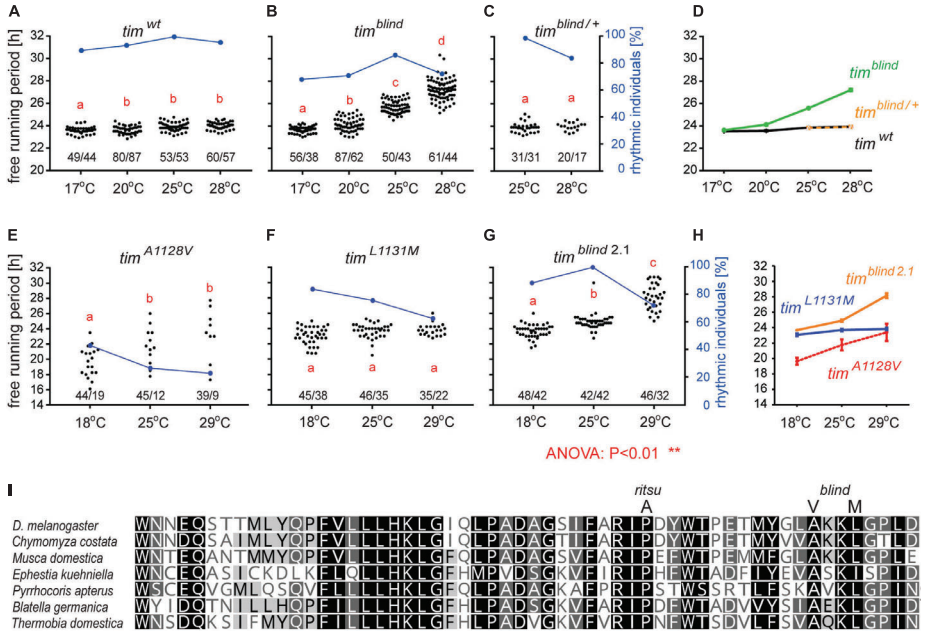


**FIGURE 1 |** *timeless*$^{blind}$ is a circadian clock mutant with defect in temperature compensation [each dot in panels **A–C** and **E–G** represents the free running period (*tau*, τ) of individual male flies recorded during 10 days in DD at the specified temperature]. Blue dots connected with a line indicate the percentage of rhythmic individuals (scale is on the right *y*-axis) at a particular temperature). **(A)** τ of control Canton S wild-type (wt) flies, **(B)** *tim*$^{blind}$ homozygous, and **(C)** *tim*$^{blind/+}$ heterozygous *D. melanogaster* males in constant darkness (DD) and constant temperature (17, 20, 25, and 28°C). **(D)** Comparison of τ (shown as mean ± SEM) for all three genotypes indicates the temperature-dependent τ lengthening in *tim*$^{blind}$ and confirms that the mutant is fully recessive. **(E)** *tim*$^{A1128V}$, a mutant with only the first *tim*$^{blind}$ position mutated, is mostly arrhythmic with a potential temperature compensation defect in rhythmic individuals, **(F)** *tim*$^{L1131M}$, a mutant with only the second *tim*$^{blind}$ position mutated, exhibits normal and fully temperature-compensated τ. **(G)** The synthetic *tim*$^{blind2.1}$ allele shows temperature compensation defects and solid rhythmicity. **(H)** Comparison of τ (shown as mean ± SEM) for re-engineered single and double-mutant versions of *tim*$^{blind}$. **(I)** Alignment of various insect TIM proteins near TIM$^{rit}$ and TIM$^{blind}$ mutations (specific substitution in *rit* and *blind* is shown above the alignment). Insects are ordered according to their phylogenetic relationship from the most diverged at the top to the most ancestral at the bottom, with the following insect groups: Diptera (*Drosophila*, *Chymomyza* and *Musca*), Lepidoptera (*Ephestia*), Heteroptera (*Pyrrhocoris*), Blattodea (*Blatella*), and Ametabola (*Thermobia*). A, V, and M refer to the single letter aminoacid code.

temperature compensation, and we show here that both amino acid substitutions are required to elicit this phenotype.

Interestingly, in both *tim* temperature compensation alleles, *tim*$^{rit}$ and *tim*$^{blind}$, the mutations are located in the same region of the TIM protein, separated by only 11–14 amino acids (**Figure 1I**). TIM$^{rit}$ was isolated from a natural fruitfly population and harbors a proline (P) to alanine (A) amino acid substitution at position 1093 (Matsumoto et al., 1999) which corresponds to position 1116 in the L-TIM protein, where additional 23 amino acids are added at the N-terminus (Tauber et al., 2007). Protein alignment indicates that these TIM regions are highly conserved across even distantly related insect species, including hemimetabolan *P. apterus*, *B. germanica*, and ametabolan *T. domestica* (**Figure 1I**).

The most common NES motif that is recognized by CRM1 is best described by the consensus sequence L-X(2,3)-[LIVFM]-X(2,3)-L-X-[LI], where X(2,3) represents any two or three amino acids that separate the four key hydrophobic residues. Although a large amount of proteins contain this consensus sequence, only a small percentage of NESs are actually functional. On the other hand, only 36% of experimentally identified functional NESs match this consensus (Kosugi et al., 2008). Thus, predicting functional NES motives is still challenging and a reliable approach has not yet been described. Therefore, we used different approaches to analyze potential NES motives in TIM.

First, we assess the evolutionary conservation of NES in TIM by identifying and comparing NES motifs in the above-mentioned "primitive" insect species and included two additional dipteran (*C. costata* and *M. domestica*) and one lepidopteran (*E. kuehniella*) representatives. We searched for strict NES motifs using the consensus after Ashmore et al. (2003). Six NES motifs were found in the entire *Drosophila* TIM, and two of them located near the TIM$^{blind}$ and TIM$^{rit}$ mutations. The NES 1031-1139, which overlaps with TIM$^{blind}$, is further conserved in all Diptera

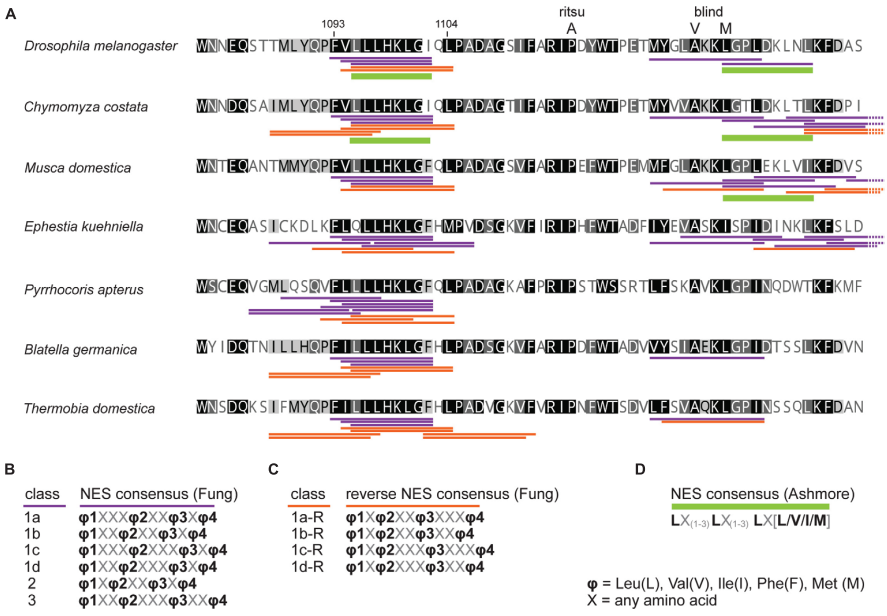


**FIGURE 2 |** *In silico* prediction of nuclear export signals (NESs) in TIM$^{rit}$ vicinity. **(A)** Protein alignment (ancestral species are at the bottom) with underlined NES motifs. Purple and orange colors code for forward and reverse NES classes, respectively, and green bar indicates strict NES prediction after Ashmore et al. (2003). **(B–D)** The consensus sequences of NES classes. Numbers above the alignment correspond to amino acid residues in L-TIM (*D. melanogaster*).

(**Figure 2**). The second NES, located upstream of TIM$^{rit}$ (residues 1095-1012), is apart from *D. melanogaster* only found in the drosophilid fly *C. costata*. To identify additional putative NES in the TIM$^{blind}$ and TIM$^{rit}$ regions, we applied less strict motifs as described in Fung et al. (2015). Multiple NES motifs were found in *D. melanogaster* near the TIM$^{blind}$ and TIM$^{rit}$ mutations, all of them at least partially overlapping with the two strict "Ashmore's" NES (**Figure 2A**). Using the less rigid "Fung" consensus, NES motifs were even found in species for which the strict consensus did not reveal any NES. Importantly, these less strict NES motifs are still present in homologous sequences (**Figure 2**).

We hypothesized that the fact that both *tim* temperature compensation mutants, *tim$^{rit}$* and *tim$^{blind}$*, are spaced close to each other is not merely accidental, as all the other known *tim* mutations that are dispersed throughout the *tim* gene region have not been reported to have a temperature-dependent phenotype. To test this assumption, we decided to conduct a targeted mutagenesis screen for temperature compensation mutations in *tim*.

## Step-Up Protocol for Detection of Temperature Compensation Mutants

To unambiguously determine and compare τ at different temperatures in individual flies, we developed and optimized a protocol for assaying locomotor activity in flies exposed to two different temperatures. Fruit flies can survive for more than 2 weeks in the glass tubes (5 mm diameter, 70 mm length, and at least 1 cm of agar with 5% sucrose) used in DAM2 monitors, so we decided to record their activity at low temperature for 7 days followed by further 7 days at high temperature providing enough data for each condition in a single fly (**Figures 3A,B,D**). The transition from the low to the high temperatures was experimentally optimized to an 8°C increase spread over 24 h (this gradual temperature rise was programmed in MIR 154, Panasonic, as eight successive steps, each 2 h long with 1°C increase) (**Figures 3A,B,D**). We verified that the τ values measured in this step-up protocol are comparable to values obtained at constant temperatures (**Figures 3E–G**). Representative actograms in **Figure 3C** illustrate that even the relatively subtle τ change of *tim$^{blind}$* can be easily spotted by eye and so that this approach is suitable for efficient screening of large datasets for altered circadian phenotypes.

## CRISPR/CAS9 Targeted Mutagenesis of *tim*

Seven regions of TIM were selected for targeted mutagenesis based on phylogenetic conservation analyses and/or their position with respect to known temperature compensation

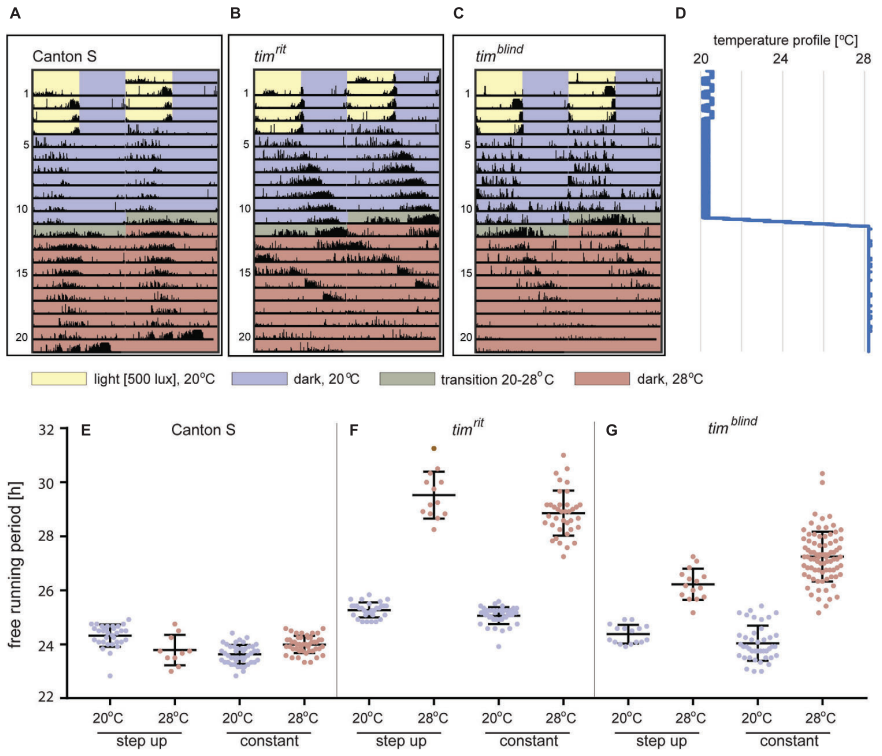


**FIGURE 3 |** Locomotor activity of *D. melanogaster* at different temperatures. **(A–C)** Typical double-plotted actograms obtained during the step-up protocol consisting of 4-day LD entrainment at 20°C followed by 7 days of DD at 20°C. Then, temperature was raised to 28°C during 16 h (1°C every 2 h) and kept constant for the rest of the experiment. **(D)** Temperature profile recorded during the step-up protocol (note small thermoperiodic oscillations typical for LD conditions). **(E–G)** Comparison of the free running periods (τ) measured at low and high portions of the step-up protocol, respectively, with data obtained from flies entrained and recorded at one constant temperature. Each dot corresponds to FRP of individual male fly, and colors correspond to the temperature-coding used in actogram backgrounds. Not all males survived entire step-up experiment (note lower *n* at 28°C). Bars show the mean ± SD.

mutations (**Figure 4**): (a) a conserved sequence motif located in the first quarter of the protein near the UL mutation, (b) $TIM^{unspliced}$ comprising the last intron which is alternatively retained at low temperatures (Boothroyd et al., 2007; Montelli et al., 2015), (c) the area where $TIM^{rit}$ and $TIM^{blind}$ mutations are located, (d) $NES^{1093-1104}$, a conserved region 12-23 aa upstream of $TIM^{rit}$, (e) $NES^{1015-1023}$ motif 93-98 aa upstream of $TIM^{rit}$, (f) $NES^{1166-1174}$ motif 56-62 aa downstream of $TIM^{rit}$, and (g) $NES^{776-785}$ located approximately in the middle of TIM (see **Table 1** for gRNA sequences, cleavage sites and corresponding sequences and **Figure 4** for position of selected regions on TIM).

Corresponding gRNA expressing plasmids were cloned, verified, and stably transformed into the attP2 landing site on the third chromosome following an established protocol (Kondo and Ueda, 2013). The targeted cleavage of genomic DNA within the *tim* gene region was induced in the embryonic stage by combining the nosCAS9 and U6gRNA-expressing transgenes to create in/dels resulting from NHEJ mechanism. The targeted chromosomes were balanced and subsequently brought into homozygosity (see **Supplementary Figure S1** for genotypes and genetic crosses and **Supplementary Figure S2** for τ of used *Drosophila* lines and lines suitable for similar experiments). In total, 618 lines covering seven *tim* regions were established and screened for altered circadian rhythmicity using our step-up protocol (**Table 4**).

Both arrhythmic mutants and mutant lines displaying altered rhythmicity were identified in the screen. In total, we isolated 113 arrhythmic lines of which a subset was molecularly characterized. In all cases, this uncovered out-of-frame in/del mutations resulting in premature stop codons. In contrast, τ-altering mutations always contained in-frame modifications. Deletions were approximately 15 times more frequent than insertions, the

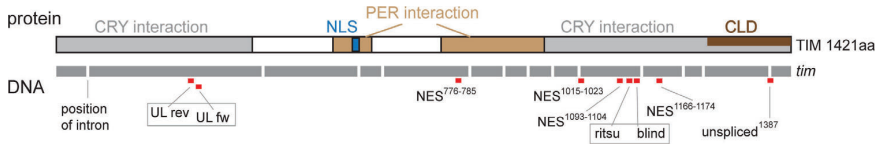


**FIGURE 4 |** Schematic depiction of TIM protein with highlighted functional domains and interacting regions: nuclear localization signal (NLS), PERIOD interacting domain (PER interaction), CRYPTOCHROME interacting domain (CRY interaction), and cytoplasmic localization domain (CLD). Below, schematic depiction of DNA is shown with exons in gray (length of the bar corresponds to exon length) separated by introns (the length is not shown). Red boxes indicate position of gRNA; the number in gRNA corresponds to amino acid residues in the TIM protein. Gray frame indicates when two gRNAs were used simultaneously in one construct (UL rev + UL fw; ritsu + blind).

length of deletions ranged from 1 to 71 bp, insertions were 2 to 8 bp long, and one-third of in/dels were combined with a substitution (see **Supplementary Figure S3** for DNA sequences of isolated mutants); 35 behaviorally normal flies were also sequenced and no modifications were observed.

## FUNCTIONAL CHARACTERIZATION OF NOVEL MUTANT LINES

The step-up protocol was efficient for quick identification of putative mutants with either arrhythmic behavior or with a change in τ including even small temperature-dependent lengthening. However, for precise phenotype assessment, follow-up experimental replicates were performed allowing us to determine the accurate percentage of rhythmicity associated with each mutation, the exact τ at four tested temperatures, and the phenotypes in heterozygous conditions (heteroallelic combinations with wild-type *tim* and $tim^{01}$).

### Region Upstream of $TIM^{UL}$

Three lines with altered τ were recovered for the region 13 aa upstream from $TIM^{UL}$ and we analyzed each of them in more detail (**Table 4**). Interestingly, all three included modification of tryptophan 270 (**Figure 5E**). The $tim^{\Delta W270}$ deletion prolonged τ by ∼1.8 h and the single aa substitution $tim^{W270Y}$ resulted in 0.6–1 h shortening of the free-running period. The effect was temperature independent in both cases and both lines were robustly rhythmic (>80% of rhythmic males at any temperature tested) (**Figures 5A,A',B,B',D**). In contrast, substitution of the tryptophan with three tyrosines ($tim^{W270YYY}$) produced temperature-dependent lengthening of τ and severe arrhythmicity in homozygotes (see blue dots connected by line; scale on right *y*-axis); however, a comparable drop in rhythmicity was not observed in heteroallelic combinations with $tim^{01}$ (**Figures 5C,C',D,D'**). For statistical comparison of τ in homozygotes, heteroallelic combinations with $tim^{01}$, and wild-type flies, see **Supplementary Figure S4**.

Furthermore, according to NetPhos 3.1 predictions, these mutations may impact the phosphorylation of the nearby serines in position 268 and 274, and although this needs to be experimentally tested, it presents an intriguing possible explanation of the functional significance of this region (**Figure 5E**). In the wild-type protein ($TIM^{wt}$), serine 268 is not likely to be phosphorylated (score 0.47, below the threshold 0.5), whereas the W270 deletion raises the prediction score to 0.78 and substitution by three tyrosines ($tim^{W270YYY}$) to 0.55. Similarly, serine at position 274 has a phosphorylation prediction score of 0.56, just above the threshold, but this is substantially increased by the presence of either $tim^{\Delta W270}$, $tim^{W270Y}$, or $tim^{W270YYY}$ (to 0.94, 0.82, or 0.92, respectively).

### Regions in Vicinity of $TIM^{rit}$ and $TIM^{blind}$ Mutations

An interspecific comparison of insect TIM proteins identified a conserved region near and especially upstream of the $TIM^{blind}$ and $TIM^{rit}$ mutations. This includes a putative NES overlapping with $TIM^{blind}$ and a second putative NES, FVLLLHKLGIQL (residues 1093-1104), located 12-23 aa upstream of $TIM^{rit}$. Both NESs were also predicted by the strict ("Ashmore") and the less strict ("Fung") consensus searches (**Figure 2**). Again, we probed the functional significance of this region by inducing NHEJ-mediated mutagenesis followed by locomotor activity screening. Four different mutants with abnormal τ were recovered for $NES^{1093-1104}$ consisting of 2 to 11 aa long deletions (**Figure 7D**). In all four cases, τ gradually increased with rising temperature and was significantly longer compared to controls at 25°C and even more so at 28°C. For statistical comparison of τ in homozygotes, heteroallelic combinations with $tim^{01}$, and wild-type flies, see **Supplementary Figure S5**. Furthermore, the percentage of rhythmicity was severely reduced in three of these mutants at 28°C with $tim^{\Delta 1092-97:PFVLLL;K1099L}$ being completely arrhythmic both as homozygotes and in heteroallelic combination with $tim^{01}$ (**Figures 6A–D,A'–D'**). This strongly contrasts with the relatively robust rhythmicity (>75%) observed in all four mutants at 25°C. Detailed sequence analysis revealed various degrees of $NES^{1093-1104}$ motif disruption in all four mutants (**Figure 8A**). Five overlapping NES classes ("Fung") and one strict motif ("Ashmore's") can be found within the region 1093-1104 in wild-type TIM and all of them are completely lost in $tim^{\Delta LYQPFVLLLHK1089-99}$, while $tim^{\Delta 1092-97:PFVLLL;K1099L}$ has one putative "Fung's" NES class remaining, while in $tim^{\Delta FV1093-94}$ and $tim^{\Delta PFVLL1092-96}$, two $NES^{1093-1104}$ according to "Fung" classes and one strict motif remain. Although all four $NES^{1093-1104}$ mutants are

**TABLE 4 |** Summary of screened lines and identified mutants.

| TIM region/targeted motif | gRNA | Lines analyzed (*n*) | Arrhythmic strains (*n*) | Altered τ (*n*) | Unique strains with altered τ (*n*) | Mutation frequency (%) |
|---|---|---|---|---|---|---|
| Upstream of UL SLWFEASLS SNTSPPKQ | UL rev | 122 | 26+1 | 5 | 3 | 26.22 |
| | UL fw | 122 | 2+1 | 0 | 0 | 2.45 |
| NES$^{776-785}$ LLLILDSSA | fw 781-789 | 72 | 8 | 0 | 0 | 11.11 |
| NES$^{1015-1023}$ ILLDLIIKE | fw 1017-1025 | 67 | 9 | 1 | 1 | 14.92 |
| NES$^{1093-1104}$ YQPFVLLLH | rev 1090-1098 | 79 | 27 | 7 | 6 | 43.03 |
| ritsu PDYWTPET blind MYGLAKKLG | rev ritsu | 130 | 30+1 | 26+3 | 7+1 | 43.84 |
| | fw-blind | 130 | 1 | 3 | 1 | 3.08 |
| NES$^{1166-1174}$ SLDVDLGDT | rev 1171-1179 | 73 | 8 | 2 | 2 | 13.70 |
| TIM$^{unspliced}$ EKEKEL$^{Kstop}$ | fw cold | 75 | 0 | 0 | 0 | 0 |
| total: | 9 gRNAs | 618 | 112 | 41 | 20 | 24.75 |

*Gray background refers to situation when two gRNAs were used simultaneously (double gRNA construct). Red color highlights mutants resulting from cleavage directed by both gRNAs. Mutation frequency refers to mutants with altered circadian phenotypes, whereas actual frequency of mutations at DNA level was not determined. Superscript indicates protein sequence resulting from retained intron.*

phenotypically very similar, the degree of NES modification is quite different. The common feature of all mutants is the absence of the 1c-R class NES consensus (**Figure 8A**).

Another mutant completely removing the NES$^{1124-1139}$, *tim*$^{1118AR\Delta 12}$, overlaps with the TIM$^{blind}$ mutations. However, this mutant also contains a substitution of tyrosine 1118 (a residue which is predicted to be phosphorylated) with alanine and arginine. Moreover, another predicted phosphorylation target, Y1125, is also missing in *tim*$^{1118AR\Delta 12}$ making it hard to interpret the relative importance of any of these sites for the observed phenotype (**Figure 8**). Similarly to the NES$^{1093-1104}$ mutants, *tim*$^{1118AR\Delta 12}$ produces a longer τ at 25°C and severe (nearly complete) arrhythmicity at 28°C (**Figures 6E,E'**). We further addressed the role of the two TIM$^{blind}$ residues and corresponding NES in the two re-engineered mutants. All predicted NES motifs near TIM$^{blind}$ remained intact in TIM$^{A1128V}$, yet this mutation produces mostly arrhythmic individuals and the few rhythmic flies show a potential temperature compensation defect (**Figure 1E**). In contrast, TIM$^{L1128M}$ which affects the strict ("Ashmore") NES motif is robustly rhythmic and perfectly temperature-compensated (**Figure 1F**). Notably, both the TIM$^{blind}$ and TIM$^{A1128V}$ mutations enhance the phosphorylation prediction score of Y1125 (**Figure 8A**). However, it is unknown, if Y1125 is phosphorylated *in vivo*.

Several τ-altering mutants mapping close to the TIM$^{rit}$ mutation were recovered (**Figure 7D**). The most severe phenotype was seen in *tim*$^{\Delta FARIPD1112-1117}$ (**Figures 6H,H'**, **7B,B'**) with overall low rhythmicity across temperatures and complete arrhythmicity at 28°C. Free running period is ∼30 h at 25°C for homozygotes, the longest τ of all mutants recovered in this region. However, τ was significantly shorter (∼27 h) in heteroallelic combinations with *tim*$^{01}$ at 25°C (**Supplementary Figure S5**). A slightly less severe impact on rhythmicity was found in *tim*$^{\Delta PDYWT1116-20}$, which is rhythmic at all temperatures, although rhythmicity at 28°C drops below 20% in homozygotes, whereas two-third of heteroallelic combinations with *tim*$^{01}$ remain rhythmic (**Figures 6G,G'**). The flies also display loss of temperature compensation as their free-running period gets longer at high temperatures. For comparison, the proline 1116 to alanine substitution in *tim*$^{rit}$ produces gradually longer τ at 25 and 28°C with rhythmicity above 80% at all temperatures tested (**Figures 6I,J,L**). The smallest, yet significant extension of τ was observed in *tim*$^{\Delta Y1118}$ (**Figure 6F**), a mutant lacking tyrosine 1118, which is likely phosphorylated (score 0.68, **Figure 8A**). Phosphorylation score of Y1118 is reduced in TIM$^{rit}$ to 0.52 and this residue is deleted, together with the surrounding four amino acids, in *tim*$^{\Delta PDYWT1116-20}$. The mutant with the strongest phenotype, *tim*$^{\Delta FARIPD1112-1117}$, has a lower predicted phosphorylation score for Y1118, identical with the score calculated in TIM$^{rit}$. In addition, deletion of residues 1112-1117 (FARIPD) also changes the phosphorylation score of the upstream S1110 from a non-significant level to 0.91.

Systematic targeting of the TIM$^{rit}$ and TIM$^{blind}$ region and the conserved region 12-23 aa upstream of TIM$^{rit}$ encompassing a putative NES$^{1093-1104}$ motifs (FVLLLHKLGIQL) resulted in mutants showing various degrees of arrhythmicity and temperature-dependent τ increase. To elucidate whether the above-mentioned region including TIM$^{rit}$, TIM$^{blind}$, and NES$^{1093-1104}$ is uniquely important for temperature compensation of the circadian clock, comparable mutagenesis was performed in regions with NES motifs located ∼50 aa downstream (NES$^{1166-1174}$) and ∼50 aa upstream (NES$^{1015-1023}$). The mutagenesis was successful in both regions, which is demonstrated by our ability to isolate 9 and 8 fully arrhythmic mutant lines, respectively (**Table 3**). In contrast, only three rhythmic mutants with altered rhythmicity or changed τ were found and although one of them, *tim*$^{\Delta DVDLG1173-77}$, produces significant lengthening of τ with rising temperature, the change is minimal, about 1 h (**Figures 6I,J,K,K',L**, **7C,C',E–G**). The deletion in *tim*$^{\Delta DVDLG1173-77}$ destroys the putative NES$^{1166-1174}$ motif and slightly changes the phosphorylation score of S1071 (from 0.49 to 0.58) and T1079 (from 0.56 to 0.50) (**Figure 8C**). A partially overlapping deletion, *tim*$^{\Delta LD1172-73}$, does not remove the NES$^{1166-1174}$, but the phosphorylation scores are similarly affected for both S1071 (from 0.49 to 0.60)

**TABLE 5 |** Summary of circadian phenotypes in new mutants and reference lines.

| | | 17°C | | | | 20°C | | | | 25°C | | | | 28°C | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TIM region** | **Genotype** | ***(n)*** | **Rhythm %** | **FRP (h)** | **SEM** | ***(n)*** | **Rhythm %** | **FRP (h)** | **SEM** | ***(n)*** | **Rhythm %** | **FRP (h)** | **SEM** | ***(n)*** | **Rhythm %** | **FRP (h)** | **SEM** |
| | wt-CS (Canton S) | 56 | 89.80 | 23.59 | 0.05 | 88 | 93.75 | 23.62 | 0.04 | 66 | 100.00 | 23.91 | 0.05 | 72 | 95.00 | 23.99 | 0.04 |
| | CS/*tim*$^{01}$ | 30 | 96.67 | 32.66 | 0.06 | 26 | 96.15 | 23.78 | 0.05 | 25 | 100.00 | 23.95 | 0.06 | 28 | 92.86 | 23.57 | 0.05 |
| Upstream of UL | *tim*$^{\Delta W270}$ | 48 | 80.43 | 25.25 | 0.05 | 56 | 93.75 | 25.41 | 0.06 | 48 | 92.50 | 25.74 | 0.10 | 48 | 86.67 | 25.55 | 0.07 |
| | *tim*$^{\Delta W270}$/+ | 32 | 93.55 | 24.62 | 0.07 | 32 | 100.00 | 24.22 | 0.05 | 32 | 100.00 | 24.45 | 0.04 | 32 | 76.67 | 24.74 | 0.08 |
| | *tim*$^{\Delta W270}$/*tim*$^{01}$ | 32 | 100.00 | 25.31 | 0.05 | 32 | 100.00 | 25.69 | 0.06 | 32 | 96.43 | 25.86 | 0.07 | 32 | 96.67 | 26.32 | 0.09 |
| | *tim*$^{W270Y}$ | 32 | 90.32 | 23.04 | 0.06 | 48 | 97.87 | 22.97 | 0.05 | 40 | 92.50 | 22.95 | 0.05 | 47 | 97.87 | 23.13 | 0.05 |
| | *tim*$^{\Delta W270Y}$/+ | 32 | 93.75 | 22.89 | 0.06 | 32 | 93.33 | 22.73 | 0.05 | 32 | 100.00 | 23.34 | 0.06 | 32 | 79.31 | 22.93 | 0.08 |
| | *tim*$^{\Delta W270Y}$/*tim*$^{01}$ | 32 | 100.00 | 22.91 | 0.04 | 32 | 96.15 | 23.21 | 0.05 | 32 | 96.77 | 23.30 | 0.08 | 64 | 96.36 | 23.33 | 0.07 |
| | *tim*$^{W270YYY}$ | 56 | 21.82 | 24.48 | 0.14 | 48 | 53.49 | 24.81 | 0.07 | 56 | 19.61 | 25.14 | 0.28 | 48 | 11.90 | 26.95 | 0.43 |
| | *tim*$^{\Delta W270YYY}$/+ | 32 | 93.10 | 24.19 | 0.08 | 32 | 100.00 | 24.03 | 0.05 | 32 | 96.67 | 24.03 | 0.09 | 32 | 93.33 | 24.52 | 0.16 |
| | *tim*$^{\Delta W270YYY}$/*tim*$^{01}$ | 32 | 93.33 | 24.55 | 0.05 | 32 | 100.00 | 24.73 | 0.06 | 32 | 100.00 | 25.61 | 0.07 | 32 | 82.76 | 26.61 | 0.17 |
| NES$^{1017-1025}$ | *tim* $^{\Delta I1022}$ | 48 | 87.23 | 23.04 | 0.06 | 48 | 97.73 | 23.34 | 0.04 | 16 | 100.00 | 23.23 | 0.05 | 32 | 80.65 | 23.27 | 0.10 |
| | *tim* $^{\Delta I1022}$/+ | 32 | 96.55 | 23.39 | 0.06 | 64 | 95.16 | 23.42 | 0.03 | 48 | 93.62 | 23.29 | 0.07 | 32 | 93.55 | 23.01 | 0.08 |
| | *tim* $^{\Delta I1022}$/*tim*$^{01}$ | 32 | 93.75 | 23.74 | 0.04 | 32 | 93.75 | 23.70 | 0.04 | 32 | 100.00 | 23.42 | 0.07 | 32 | 93.33 | 23.11 | 0.07 |
| NES$^{1093-1104}$ | *tim*$^{\Delta LYQPFVLLLHK1089-99}$ | 40 | 47.37 | 24.56 | 0.07 | 48 | 43.48 | 24.77 | 0.18 | 48 | 81.82 | 28.09 | 0.14 | 48 | 12.77 | 30.75 | 0.71 |
| | *tim*$^{\Delta LYQPFVLLLHK1089-99}$/+ | 48 | 90.48 | 23.27 | 0.05 | 32 | 96.67 | 23.54 | 0.06 | 48 | 93.75 | 24.01 | 0.06 | 32 | 90.00 | 23.88 | 0.05 |
| | *tim*$^{\Delta LYQPFVLLLHK1089-99}$/*tim*$^{01}$ | 32 | 84.38 | 23.82 | 0.10 | 32 | 82.76 | 24.19 | 0.10 | 40 | 94.87 | 26.38 | 0.17 | 32 | 31.25 | 30.61 | 0.38 |
| | *tim*$^{\Delta PFVLL1092-96}$ | 56 | 50.94 | 25.13 | 0.10 | 48 | 64.58 | 24.61 | 0.08 | 56 | 86.79 | 26.29 | 0.06 | 48 | 45.83 | 30.72 | 0.16 |
| | *tim*$^{\Delta PFVLL1092-96}$/+ | 32 | 84.38 | 23.45 | 0.05 | 32 | 93.33 | 23.31 | 0.05 | 32 | 96.88 | 23.66 | 0.04 | 32 | 100.00 | 24.74 | 0.14 |
| | *tim*$^{\Delta PFVLL1092-96}$/*tim*$^{01}$ | 49 | 89.58 | 23.71 | 0.07 | 32 | 96.43 | 23.82 | 0.08 | 48 | 97.62 | 25.48 | 0.08 | 36 | 72.73 | 28.92 | 0.29 |
| | *tim*$^{\Delta PFVLLL1092-97;K1099L}$ | 80 | 72.15 | 24.74 | 0.05 | 48 | 76.60 | 25.08 | 0.06 | 66 | 83.93 | 28.37 | 0.08 | 80 | 0.00 | | |
| | *tim*$^{\Delta PFVLLL1092-97;K1099L}$/+ | 32 | 81.25 | 23.64 | 0.02 | 32 | 81.48 | 24.10 | 0.09 | 48 | 95.24 | 24.59 | 0.06 | 32 | 92.59 | 24.56 | 0.06 |
| | *tim*$^{\Delta PFVLLL1092-97;K1099L}$/*tim*$^{01}$ | 32 | 89.29 | 24.08 | 0.13 | 32 | 78.13 | 24.68 | 0.14 | 64 | 76.67 | 27.10 | 0.16 | 32 | 0.00 | | |
| | *tim*$^{\Delta FV1093-94}$ | 32 | 81.25 | 23.61 | 0.08 | 48 | 95.74 | 24.06 | 0.05 | 48 | 82.93 | 27.66 | 0.13 | 48 | 12.77 | 31.54 | 0.05 |
| | *tim*$^{\Delta FV1093-94}$/+ | 32 | 87.50 | 23.22 | 0.05 | 32 | 100.00 | 23.49 | 0.05 | 32 | 96.67 | 23.82 | 0.07 | 32 | 96.67 | 24.86 | 0.22 |
| | *tim*$^{\Delta FV1093-94}$/*tim*$^{01}$ | 32 | 77.42 | 23.62 | 0.09 | 32 | 96.67 | 23.89 | 0.06 | 32 | 87.50 | 25.96 | 0.13 | 40 | 55.88 | 27.35 | 0.67 |
| ritsu | *tim*$^{rit}$ | 48 | 89.74 | 24.82 | 0.28 | 80 | 97.67 | 25.06 | 0.31 | 32 | 93.33 | 26.08 | 0.33 | 64 | 84.75 | 28.86 | 0.82 |
| | *tim*$^{rit}$/+ | 28 | 100.00 | 24.11 | 0.06 | 15 | 100.00 | 24.03 | 0.03 | 16 | 100.00 | 24.28 | 0.06 | 14 | 100.00 | 24.35 | 0.03 |
| | *tim*$^{rit}$/*tim*$^{01}$ | 28 | 100.00 | 23.53 | 0.12 | 29 | 100.00 | 24.41 | 0.04 | 30 | 100.00 | 25.51 | 0.05 | 32 | 100.00 | 27.57 | 0.18 |
| | *tim*$^{\Delta FARIPD1112-17}$ | 48 | 14.58 | 24.55 | 0.22 | 48 | 42.55 | 25.13 | 0.08 | 48 | 52.08 | 30.16 | 0.14 | 56 | 0.00 | | |
| | *tim*$^{\Delta FARIPD1112-17}$/+ | 32 | 48.39 | 23.42 | 0.09 | 32 | 90.32 | 23.57 | 0.06 | 32 | 100.00 | 23.62 | 0.06 | 32 | 100.00 | 24.41 | 0.09 |
| | *tim*$^{\Delta FARIPD1112-17}$/*tim*$^{01}$ | 32 | 65.52 | 22.89 | 0.12 | 32 | 86.21 | 24.06 | 0.09 | 48 | 87.50 | 27.04 | 0.21 | 32 | 0.00 | | |
| | *tim*$^{\Delta PDYWT1116-20}$ | 48 | 71.74 | 24.44 | 0.08 | 48 | 70.21 | 24.73 | 0.10 | 32 | 79.31 | 27.42 | 0.17 | 80 | 16.00 | 30.28 | 0.36 |
| | *tim*$^{\Delta PDYWT1116-20}$/+ | 62 | 93.33 | 23.42 | 0.06 | 32 | 96.88 | 23.38 | 0.06 | 48 | 100.00 | 23.93 | 0.04 | 36 | 97.06 | 24.55 | 0.08 |
| | *tim*$^{\Delta PDYWT1116-20}$/*tim*$^{01}$ | 32 | 87.50 | 23.30 | 0.08 | 32 | 96.88 | 24.05 | 0.07 | 32 | 80.65 | 26.23 | 0.23 | 40 | 66.67 | 30.09 | 0.23 |
| | *tim* $^{\Delta Y1118}$ | 49 | 60.87 | 23.95 | 0.08 | 48 | 93.48 | 23.99 | 0.04 | 40 | 94.44 | 24.65 | 0.05 | 48 | 71.11 | 25.55 | 0.08 |
| | *tim* $^{\Delta Y1118}$/+ | 32 | 90.63 | 23.51 | 0.06 | 32 | 100.00 | 23.61 | 0.06 | 32 | 90.32 | 24.10 | 0.09 | 32 | 96.30 | 24.29 | 0.08 |

*(Continued)*

**TABLE 5 |** Continued

| | | 17°C | | | | 20°C | | | | 25°C | | | | 28°C | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TIM region** | **Genotype** | ***(n)*** | **Rhythm %** | **FRP (h)** | **SEM** | ***(n)*** | **Rhythm %** | **FRP (h)** | **SEM** | ***(n)*** | **Rhythm %** | **FRP (h)** | **SEM** | ***(n)*** | **Rhythm %** | **FRP (h)** | **SEM** |
| | $tim^{\Delta Y1118}/tim^{01}$ | 32 | 96.77 | 23.61 | 0.05 | 32 | 100.00 | 24.12 | 0.04 | 32 | 100.00 | 24.48 | 0.08 | 48 | 100.00 | 25.20 | 0.10 |
| $NES^{1166-1174}$ | $tim^{\Delta LD1172-73}$ | 48 | 50.00 | 23.43 | 0.04 | 48 | 65.22 | 23.67 | 0.05 | 16 | 40.00 | 23.57 | 0.10 | 48 | 50.00 | 23.18 | 0.06 |
| | $tim^{\Delta LD1172-73}/+$ | 32 | 83.33 | 23.46 | 0.07 | 32 | 100.00 | 23.50 | 0.06 | 32 | 100.00 | 23.29 | 0.07 | 32 | 91.67 | 23.14 | 0.12 |
| | $tim^{\Delta LD1172-73}/tim^{01}$ | 40 | 93.94 | 23.80 | 0.05 | 32 | 100.00 | 23.67 | 0.06 | 40 | 90.63 | 23.93 | 0.08 | 32 | 93.75 | 23.16 | 0.07 |
| | $tim^{\Delta DVDLG1173-77}$ | 40 | 71.79 | 23.24 | 0.06 | 56 | 80.77 | 23.67 | 0.05 | 10 | 80.00 | 23.60 | 0.10 | 32 | 48.15 | 24.26 | 0.18 |
| | $tim^{\Delta DVDLG1173-77}/+$ | 32 | 90.32 | 23.62 | 0.05 | 32 | 100.00 | 23.68 | 0.04 | 32 | 96.77 | 23.93 | 0.05 | 32 | 100 | 23.58 | 0.07 |
| | $tim^{\Delta DVDLG1173-77}/tim^{01}$ | 32 | 96.77 | 23.58 | 0.05 | 32 | 100.00 | 24.33 | 0.05 | 32 | 96.88 | 24.20 | 0.06 | 64 | 94.643 | 24.75 | 0.11 |
| blind | $tim^{1118AR;\ \Delta\ 12aa}$ | 112 | 64.52 | 24.95 | 0.09 | 48 | 70.73 | 25.24 | 0.18 | 64 | 68.33 | 27.92 | 0.15 | 88 | 3.70 | 29.42 | 1.00 |
| | $tim^{1118AR;\ \Delta\ 12aa}/+$ | 32 | 66.67 | 23.68 | 0.10 | 32 | 83.87 | 23.81 | 0.09 | 80 | 100.00 | 24.58 | 0.04 | 48 | 97.83 | 24.72 | 0.08 |
| | $tim^{1118AR;\ \Delta\ 12aa}/tim^{01}$ | 32 | 73.08 | 24.21 | 0.15 | 32 | 73.68 | 25.20 | 0.15 | 40 | 66.67 | 28.10 | 0.20 | 32 | 0.00 | | |
| | $tim^{blind}$ | 83 | 72.29 | 23.71 | 0.05 | 104 | 70.77 | 24.04 | 0.08 | 56 | 86.00 | 25.65 | 0.08 | 80 | 72.13 | 26.80 | 0.27 |
| | $tim^{blind}/+$ | 30 | 100.00 | 23.85 | 0.09 | 11 | 100.00 | 23.63 | 0.09 | 32 | 100.00 | 23.89 | 0.08 | 23 | 85.00 | 23.99 | 0.09 |
| | $tim^{blind}/tim^{01}$ | 30 | 83.33 | 23.18 | 0.08 | 30 | 100.00 | 23.93 | 0.03 | 30 | 100.00 | 25.00 | 0.05 | 30 | 96.67 | 26.19 | 0.13 |
| | | 18°C | | | | | | | | 25°C | | | | 29°C | | | |
| blind | $tim^{A1128V}$ | 44 | 43.18 | 21.67 | 1.02 | – | – | – | – | 45 | 26.67 | 22.54 | 0.97 | 39 | 23.07 | 24.70 | 0.92 |
| | $tim^{blind\ 2.1}$ | 48 | 87.50 | 23.74 | 0.91 | – | – | – | – | 42 | 100.00 | 24.88 | 0.13 | 46 | 69.57 | 27.61 | 0.16 |
| | $tim^{L1131M}$ | 45 | 84.44 | 23.22 | 0.19 | – | – | – | – | 46 | 76.09 | 23.70 | 0.16 | 35 | 62.86 | 23.82 | 0.17 |

*Rhythm % indicates percentage of rhythmic males.*

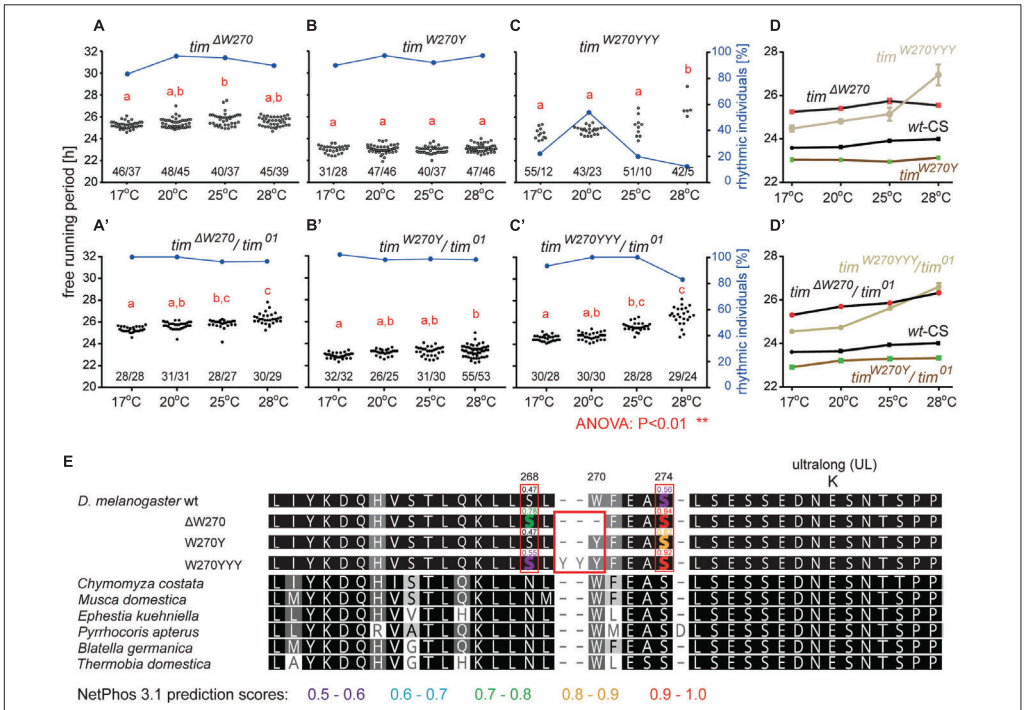


**FIGURE 5 |** Mutants isolated upstream of $tim^{UL}$. **(A–C)** Each dot represents τ of individual fly as was recorded during 10 days in DD and specified temperature. Lowercase red letters above dots indicate a significant difference between "a" and "b" and "c" and "d" (one-way ANOVA with Tukey–Kramer's *post hoc* test, $P < 0.01$). Numbers below dots correspond to number of measured/rhythmic flies at particular temperature. Blue dots connected with line correspond to percentage of rhythmic individuals (scale on right *y*-axis) at particular temperature. Panels **A–C** contain phenotypes of homozygous flies, whereas **A'–C'** are dedicated to heteroallelic combinations with $tim^{01}$. Comparison of τ (shown as mean ± SEM) for all three mutants and wild-type (Canton strain) flies (wt-CS) for **(D)** homozygotes and **(D')** heteroallelic combinations with $tim^{01}$. **(E)** Alignment of mutant protein sequences with TIM from representative insects (see the text and legend of **Figure 1** for more details on insect taxons). Mutation in position 270 (red box) possibly affects phosphorylation of serine residues in positions 268 and 274 (highlighted by red boxes). Only residues which phosphorylation is possibly affected by mutation are highlighted in the alignment of mutants and wt TIM proteins and prediction scores are indicated above the amino acid. The colors correspond to score prediction from NetPhos 3.1 (color coding is shown below the alignment).

and T1079 (from 0.56 to 0.53). This mutant is characterized by a minimal shortening of τ at 28°C (0.8 h shorter than wt, **Supplementary Figure S5J**). Finally, the $tim^{\Delta I1022}$ mutant produces a 0.3–0.75 h shorter τ than wt flies at all temperatures (**Figures 7C,C'** and **Supplementary Figure S5I**). This single amino acid deletion removes the $NES^{1015-1023}$ motif (**Figure 8B**).

## Immunocytochemistry

In order to elucidate the impact of the newly isolated *tim* mutations on the temporal clock protein expression pattern in the circadian clock neurons, we performed ICC on whole mount fly brains. Since no TIM antibody was available, we used PER immunostainings as a proxy to visualize the progression of the PER-TIM negative feedback loop. Two mutants covering the *rit* ($tim^{\Delta 1092-97:PFVLLL;K1099L}$) and *blind* ($tim^{1118AR\Delta 12}$) region were selected. Both mutants show relatively robust rhythmicity at 17–25°C, but are completely arrhythmic at 28°C (**Figures 6C,E**). In both, wt and mutants, the PER level was cycling during the day at every temperature, but in mutants, the amplitude of the oscillation was reduced (**Figure 9** and **Supplementary Figure S6**). At 17°C, PER intensity was almost comparable in small ventrolateral (s-LNv) neurons between wt, $tim^{\Delta 1092-97:PFVLLL;K1099L}$, and $tim^{1118AR\Delta 12}$, with a peak at ZT0 and a trough around ZT12. A similar trend was observed in the large ventrolateral (l-LNv) neurons, with the difference that the relative staining intensity was about half the intensity detected in s-LNv (**Figure 9D**). At 25°C, a clear difference in the intensity of the PER signal was observed between wt and the two *tim* mutants in both s-LNv and l-LNv in all ZTs with the exception of ZT12 (**Figures 9B,E** and **Supplementary Figures S6B,C**). At 28°C, the highest level of PER was detected in wt, lower levels in $tim^{\Delta 1092-97:PFVLLL;K1099L}$

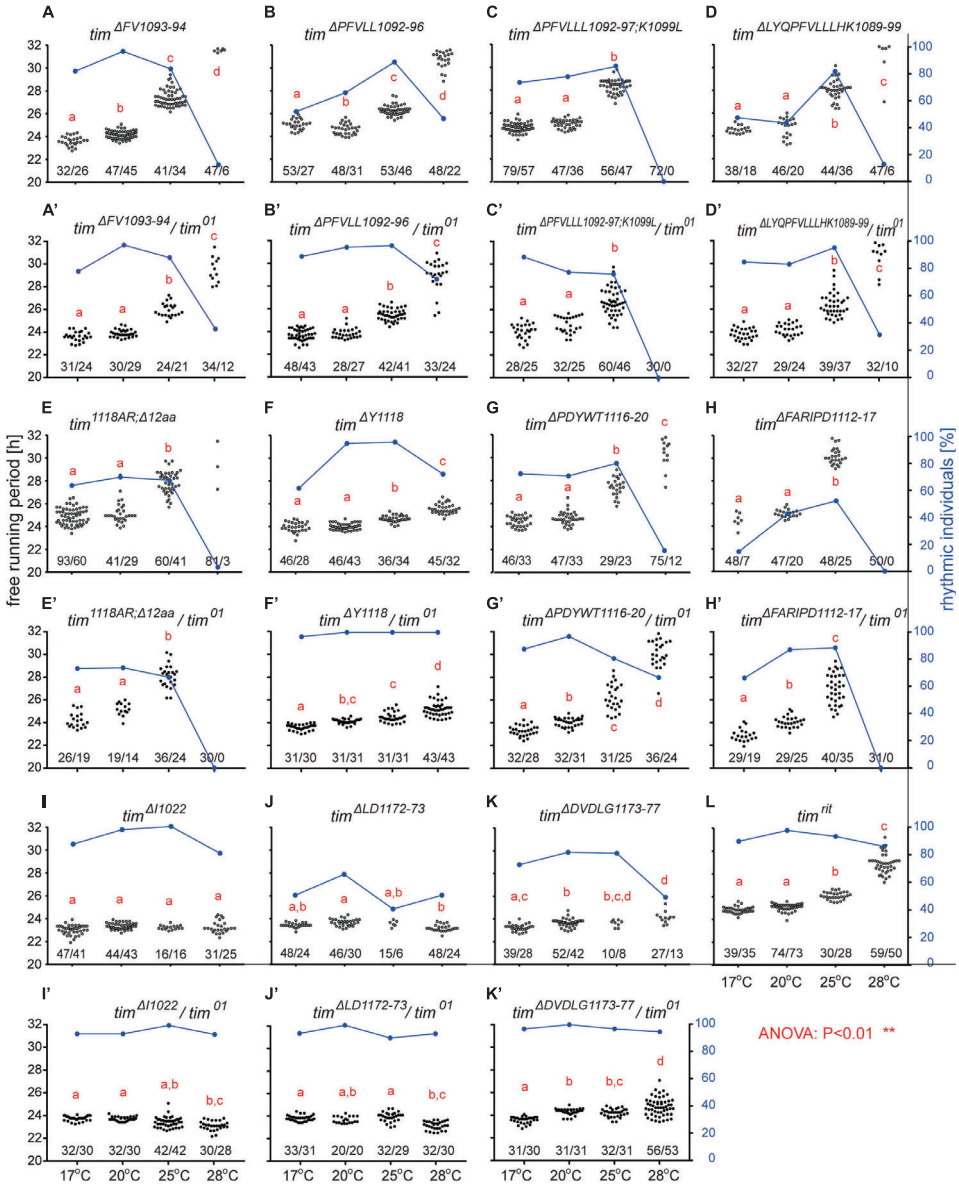


**FIGURE 6 |** Mutants isolated in vicinity of $tim^{rit}$ and $tim^{blind}$. **(A–L)** Each dot represents τ of individual fly as was recorded during 10 days in DD and specified temperature. Lowercase red letters above dots indicate a significant difference between "a" and "b" and "c" and "d" (one-way ANOVA with Tukey–Kramer's *post hoc* test, $P < 0.01$). Numbers below dots correspond to number of measured/rhythmic flies at particular temperature. Blue dots connected with line correspond to percentage of rhythmic individuals (right *y*-axis) at particular temperature.

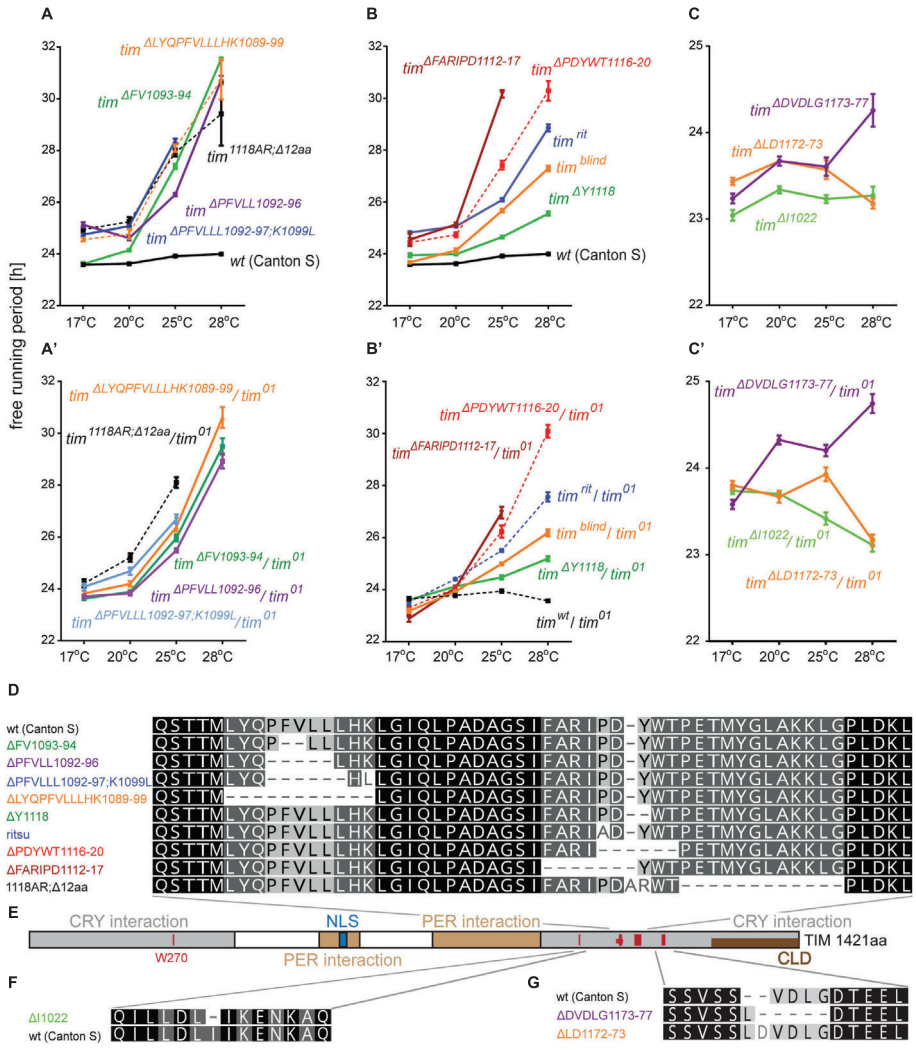


**FIGURE 7 |** Summary of phenotypes for homozygous **(A–C)** and heteroallelic combinations with $tim^{01}$ **(A'–C')** mutants isolated in vicinity of $tim^{rit}$ and $tim^{blind}$. Name of each mutation is color-coded to ease orientation in the sequence alignments **(D,F,G)**. **(E)** Schematic depiction of TIM protein with position of mutated regions (red bars), nuclear localization signal (NLS), PERIOD interacting domain (PER interaction), CRYPTOCHROME interacting domain (CRY interaction), and cytoplasmic localization domain (CLD). Dashes (–) correspond to gaps in the alignment (which result from insertions or deletions). For statistical comparison between mutants and heteroallelic combinations, see **Supplementary Figure S5**.

and the lowest level was detected in $tim^{1118AR\Delta 12}$ at ZT0, ZT4, and ZT20 (**Figures 9C,F**, **Supplementary Figure S7**). The pattern of immunostaining intensity at 17 and 25°C is thus consistent with the locomotor activity phenotypes observed in the mutants. However, both mutants are virtually arrhythmic at 28°C, yet the expression levels of PER were clearly different between them at the beginning of the day and at the end of the night.

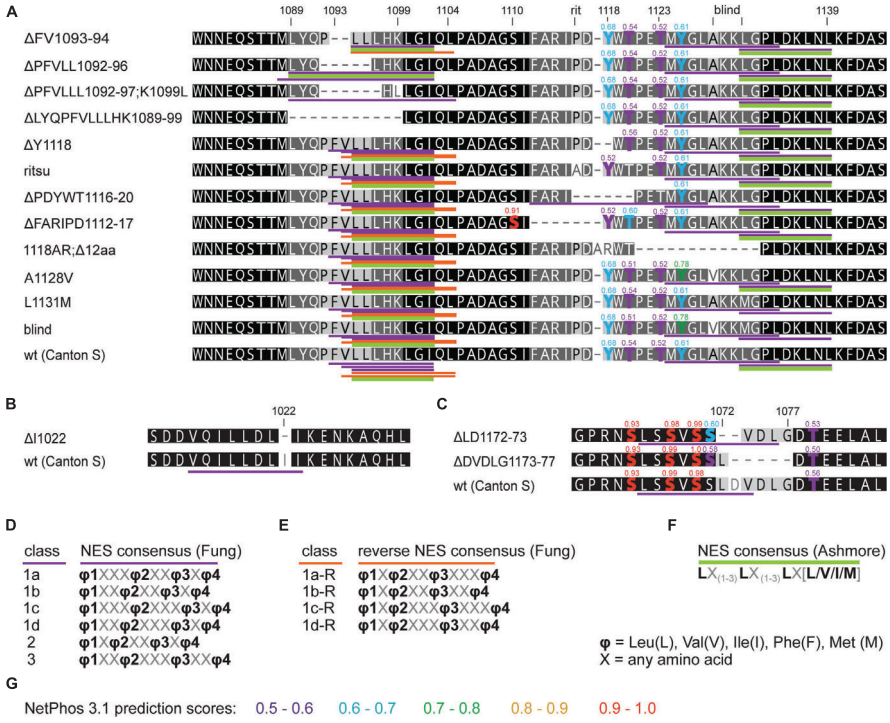


**FIGURE 8 |** *in silico* prediction of nuclear export signals (NESs) and phosphorylation in *tim* mutants. Protein alignments of **(A)** *ritsu*, *blind*, and NES 1093-1104 regions, **(B)** NES 1015-1023, and **(C)** NES 1166-1174 with underlined NES motifs. Purple, orange, and green colors code for NES classes according to consensus shown in **D–F**. Residues which phosphorylation is possibly affected by mutation are highlighted in the alignment and scores are indicated above the amino acid. **(G)** The colors correspond to prediction from NetPhos 3.1.

The experimental set-up allowed us to perform semi-quantitative comparisons between temperatures within each genotype. In s-LNv neurons of wt flies, the lowest signal intensities were observed at 17°C, whereas expression levels were more or less comparable at 25 and 28°C. In l-LNv neurons, signal intensities increased with temperature: the lowest expression was observed at 17°C, intermediate levels at 28°C, and the maximal expression at 25°C. In both mutants, expression levels varied much less across temperatures due to lower levels detected at 25 and 28°C.

## DISCUSSION

### Targeted Screen for Temperature Compensation Mutants

Circadian clock research is a remarkably successful field of experimental biology and the molecular mechanisms that build up circadian oscillators are well understood, especially in *Drosophila*. Despite these huge successes, there are a number of distressingly large gaps in our understanding of biological timekeeping. One of the key features of circadian clocks is their ability to keep a largely unchanged pace regardless of temperature, a phenomenon termed temperature compensation. To specifically generate new mutants with temperature compensation defects, we utilize the efficacy of the CRISPR/CAS9 technology as a tool for targeted mutagenesis. Simple genetic crosses are used to establish homozygous mutants and their τ is determined in a temperature step-up protocol to specifically identify mutants even if they only exhibit very subtle impairments in temperature compensation.

Although the position where the CAS9 protein cleaves chromosomal DNA is defined by the gRNA sequence, the actual mutations resulting from subsequent NHEJ are variable and virtually unpredictable in/dels. As a consequence, various degrees of gradual amino acid deletions (see **Figures 5E**, **7D**) and phenotypic changes (see **Figures 7A–C**) were obtained. More than one-quarter of created lines were circadian mutants, but

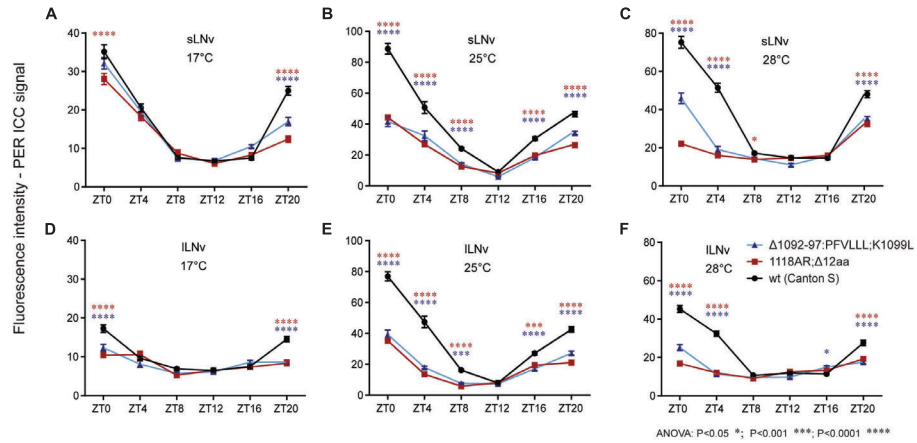


**FIGURE 9 |** Immuno-localization of PER in small and large ventrolateral neurons (s-LNv, l-LNv). Relative quantifications show role of **(A–F)** temperature on PER immunoreactivity within genotype. Only statistical difference between wt and mutants is shown for each ZT. For differences, see **Supplementary Figure S6**. s-LNv and l-LNv were plotted separately.

only 3% of screened lines were unique mutants with altered τ. Obviously, a higher percentage of mutants was probably induced, but if the phenotypic change was below our recognition, these lines were discarded. Yet, the success rate is remarkably higher than in EMS screens and importantly, identification of mutation is straightforward, fast, and cheap, compared to time demanding mapping after classical mutagenesis. The obvious limitation is that the screen presented here is strictly hypothesis-driven (Curtiz and Wallis, 1942) and mutations are created only in candidate regions of already established circadian clock genes. Therefore, this approach is suitable to saturate genes with targeted mutations and to assess the function of specific regions including coding sequence or cis-regulatory motifs in promoters.

## *Timeless* in Temperature Compensation of the Circadian Clock

This study exploited TIM in selected insect representatives. Despite the long history from the common insect ancestor (>400 million years ago), some regions of TIM are well conserved across insect species, pointing to their possible functional significance. Therefore, we experimentally tested the role of eight conserved motifs by targeted mutagenesis and isolated circadian clock mutants in seven of them. Three remarkably conserved regions located closely together, TIM$^{rit}$, TIM$^{blind}$, and NES$^{1093-1104}$, were functionally identified as particularly critical for temperature compensation. The importance of nuclear export for proper function of TIM is well established (Ashmore et al., 2003) and *tim*$^{blind}$ overlaps with NES$^{1131-1139}$ (Wülbeck et al., 2005). It is surprising that the two amino-acid replacements encoded by *tim*$^{blind}$ lead to a strong temperature compensation phenotype, because both the alanine to valine substitution at position 1128 and the leucine to methionine change at position 1131 are conservative replacements. Even more surprising is the strong impact on clock function observed in the single mutant *tim*$^{A1128V}$. The few rhythmic *tim*$^{A1128V}$ individuals are associated with variable periods but do exhibit potential period lengthening with increasing temperatures (**Figures 1E,H** and **Table 5**). It is therefore possible that in the *tim*$^{blind}$ double-mutant, the L1131M substitution, which shows no phenotype on its own, somehow suppresses the A1128V substitution, resulting in restauration of rhythmicity but maintenance of the temperature compensation phenotype. It would be very interesting to see, whether TIM$^{blind}$ residues play a similar role in *P. apterus* TIM, where neither "Ashmore's" nor "Fung's" NESs are predicted (**Figure 2A**). This type of experiment might be possible in future, as genome editing slowly becomes accessible even in non-model insects including *P. apterus* (Kotwica-Rolinska et al., 2019).

The NES$^{1093-1104}$ region described here consists of three forward and two reverses NES consensuses. However, comparably strong temperature compensation defects were observed in all mutants targeting NES$^{1093-1104}$, although different numbers of NES consensuses were depleted. Either 1c-R NES is the only essential export signal, or residues 1089-1099 have some additional role for TIM structure. Notably, all three mutants in the *tim*$^{rit}$ region mapping just 8–14 amino acids downstream from NES$^{1093-1104}$ show a temperature compensation defect, although none of these mutations directly affects NES$^{1093-1104}$. Additionally, neither mutation in NES$^{1015-1023}$ nor in NES$^{1166-1174}$ had an impact on temperature compensation. Currently, it is therefore unclear if nuclear export is indeed important for temperature compensation, or if other alterations (e.g., changes in phosphorylation), or a combination of both, contribute to the temperature compensation phenotypes

observed in several of the mutants described in this study. Analysis of the detailed subcellular localization of the mutated TIM proteins will hopefully point to the possible mechanism contributing to temperature compensation.

Our comparison often revealed a shorter τ in heteroallelic combinations of various mutants with $tim^{01}$, a combination with only one partially functional *tim* copy, when compared to homozygous mutants (**Supplementary Figure S5**). This contrasts with the dose-independent role of wild-type TIM reported previously at 25°C (Rothenfluh et al., 2000a,b; Ashmore et al., 2003) and our results obtained for $TIM^{wt}$ at all four tested temperatures (**Supplementary Figure S5A**). Notably, a ∼1 h shorter τ was also observed for $tim^{rit}/tim^{01}$ heterozygotes exposed to different temperatures (Matsumoto et al., 1999) supporting the possibility that in specific temperature conditions, the amount of a mutated TIM protein might be important for τ .

Our ICC data indicate that the PER immunostaining signal is strongly affected by *tim* mutations and that the low PER signal is more profound at high temperatures. Although the ICC data were obtained during LD, whereas circadian phenotypes were recorded in DD, PER immunostaining intensities are consistent with the behavioral defects of particular mutants. It is not clear if the stability of mutant TIM is primarily affected at 28°C, or if the interaction between PER and TIM is somehow influenced by the mutations. Therefore, PER immunostaining only serves as a proxy for the status of the PER-TIM negative feedback loop. In this regard, it is worth noting that PER and TIM do not co-localize perfectly in various neurons during the circadian cycle (Shafer et al., 2002; Wülbeck et al., 2005). Moreover, TIM also interacts with CRY in a light-dependent manner (Ceriani et al., 1999) and thus the here newly induced mutations may also affect this interaction. However, the possible change in CRY-TIM interaction should not impact free running period in DD, as CRY-depleted flies have a normal τ (Stanewsky et al., 1998; Dolezelova et al., 2007). The light resetting capacity of the new *tim* mutants is currently unknown, but given the light entrainment phenotype of $tim^{blind}$ connected with the partial resistance of $TIM^{blind}$ to light-induced degradation, it is possible that at least some of the new alleles are affected (Wülbeck et al., 2005).

The subcellular localization of TIM is connected with its phosphorylation. Long τ *tim* mutants are characterized by hypophosphorylated TIM constrained in the cytoplasm as it is known that interaction with the nuclear transport machinery is dependent on the phosphorylation state of TIM (Jang et al., 2015). Likewise, $TIM^{BLIND}$ is hypophosporylated at all times during the circadian cycle and accumulates in the cytoplasm of photoreceptor cells and LNv clock neurons, with only minor effects on PER phosphorylation and subcellular localization (Wülbeck et al., 2005). Indeed, several of our mutants between positions 1112 and 1120 affect phosphorylation predictions without altering the NES sequence. For example, deletion of tyrosine 1118 produces mild and gradual lengthening of τ at 25 and 28°C. Interestingly, a more pronounced τ extension is observed in $tim^{rit}$ mutants even though its 1116 proline to alanine substitution only reduces the phosphorylation prediction score for tyrosine 1118. The $tim^{\Delta PDYWT1116-20}$ mutation results in an even longer τ combined with substantially reduced rhythmicity at 28°C. The most severe phenotype with complete arrhythmicity at 28°C, generally low rhythmicity, and remarkable τ extension at 25°C is observed in $tim^{\Delta FARIPD1112-17}$ mutants. Interestingly, deletion of FARIPD changes the phosphorylation prediction score for serine 1110 (from a non-significant score to 0.91) in addition to mild changes in scores for tyrosine 1118 and threonine 1120. Although further functional experiments are needed to determine the actual impact of the novel mutations on TIM localization and phosphorylation, the collection of mutants presented here points to a new region of TIM important for temperature compensation.

## DATA AVAILABILITY STATEMENT

All datasets generated and analyzed for this study are included in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

SS and DD designed the study. SS performed majority of NHEJ experiments, analyzed, and interpreted the results. SF combined *per*, *tim*, and *cry* alleles and assessed their phenotypes. RS and SF independently observed the temperature compensation phenotype associated with $tim^{blind}$. AG performed HDR reengineering of $tim^{blind}$ mutants and their behavioral analysis. MD performed immunohistochemical experiments. GM contributed gRNA design and participated in early steps of the screen. DD supervised the study and together with SF wrote the manuscript with input from all co-authors.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01442/full#supplementary-material

## REFERENCES


Agrawal, P., and Hardin, P. E. (2016). An RNAi screen to identify protein phosphatases that function within the Drosophila circadian clock. *G3-Genes Genomes Genet.* 6, 4227–4238. doi: 10.1534/g3.116.035345

Arrhenius, S. (1889). Über die reaktionsgeschwindigkeit bei der inversion von Rohrzucker durch Saeuren. *Zeitschrit fuer physikalische Chemie* 4, 226–248.

Ashmore, L. J., Sathyanarayanan, S., Silvestre, D. W., Emerson, M. M., Schotland, P., and Sehgal, A. (2003). Novel insights into the regulation of the timeless protein. *J. Neurosci.* 23, 7810–7819. doi: 10.1523/jneurosci.23-21-07810.2003

Baylies, M. K., Vosshall, L. B., Sehgal, A., and Young, M. W. (1992). New short period mutations of the Drosophila clock gene per. *Neuron* 9, 575–581. doi: 10.1016/0896-6273(92)90194-i

Bazalova, O., and Dolezel, D. (2017). Daily activity of the housefly, musca domestica, is influenced by temperature independent of 3′ UTR period gene splicing. *G3-Genes Genomes Genet.* 7, 2637–2649. doi: 10.1534/g3.117.042374

Bazalova, O., Kvicalova, M., Valkova, T., Slaby, P., Bartos, P., Netusil, R., et al. (2016). Cryptochrome 2 mediates directional magnetoreception in cockroaches. *Proc. Natl. Acad. Sci. U.S.A.* 113, 1660–1665. doi: 10.1073/pnas.1518622113

Boothroyd, C. E., Wijnen, H., Naef, F., Saez, L., and Young, M. W. (2007). Integration of light and temperature in the regulation of circadian gene expression in Drosophila. *PLoS Genet.* 3:e54. doi: 10.1371/journal.pgen.0030054

Busza, A., Emery-Le, M., Rosbash, M., and Emery, P. (2004). Roles of the two Drosophila CRYPTOCHROME structural domains in circadian photoreception. *Science* 304, 1503–1506. doi: 10.1126/science.1096973

Ceriani, M. F., Darlington, T. K., Staknis, D., Mas, P., Petti, A. A., Weitz, C. J., et al. (1999). Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science* 285, 553–556. doi: 10.1126/science.285.5427.553

Chiu, J. C., Ko, H. W., and Edery, I. (2011). NEMO/NLK Phosphorylates PERIOD to initiate a time delay phosphorylation circuit that sets circadian clock speed. *Cell* 145, 357–370. doi: 10.1016/j.cell.2011.04.002

Curtiz, M., and Wallis, B. H. (1942). *Round Up the Usual Suspects. Casablanca Warner Bros.* Burbank, CA: First National Pictures.

Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D. L., et al. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. *Science* 280, 1599–1603. doi: 10.1126/science.280.5369.1599

Diernfellner, A., Colot, H. V., Dintsis, O., Loros, J. J., Dunlap, J. C., and Brunner, M. (2007). Long and short isoforms of *Neurospora* clock protein FRQ support temperature-compensated circadian rhythms. *FEBS Lett.* 581, 5759–5764. doi: 10.1016/j.febslet.2007.11.043

Dolezelova, E., Dolezel, D., and Hall, J. C. (2007). Rhythm defects caused by newly engineered null mutations in Drosophila's cryptochrome gene. *Genetics* 177, 329–345. doi: 10.1534/genetics.107.076513

Edery, I., Zwiebel, L. J., Dembinska, M. E., and Rosbash, M. (1994). Temporal phosphorylation of the Drosophila period protein. *Proc. Natl. Acad. Sci. U.S.A.* 91, 2260–2264. doi: 10.1073/pnas.91.6.2260

Fang, Y., Sathyanarayanan, S., and Sehgal, A. (2007). Post-translational regulation of the Drosophila circadian clock requires protein phosphatase 1 (PP1). *Genes Dev.* 21, 1506–1518. doi: 10.1101/gad.1541607

Fexová, S. (2010). *Circadian Clock of Two Insect Model Species - Drosophila Melanogaster and Tribolium Castaneum*. MSc thesis, University of South Bohemia, České Budějovice.

Fung, H. Y., Fu, S. C., Brautigam, C. A., and Chook, Y. M. (2015). Structural determinants of nuclear export signal orientation in binding to exportin CRM1. *eLife* 4:10034. doi: 10.7554/eLife.10034

Glaser, F. T., and Stanewsky, R. (2005). Temperature synchronization of the Drosophila circadian clock. *Curr. Biol.* 15, 1352–1363. doi: 10.1016/j.cub.2005.06.056

Glossop, N. R., Lyons, L. C., and Hardin, P. E. (1999). Interlocked feedback loops within the Drosophila circadian oscillator. *Science* 286, 766–768. doi: 10.1126/science.286.5440.766

Hamblen, M. J., White, N. E., Emery, P., Kaiser, K., and Hall, J. C. (1998). Molecular and behavioral analysis of four period mutants in Drosophila melanogaster encompassing extreme short, novel long, and unorthodox arrhythmic types. *Genetics* 149, 165–178.

Hara, T., Koh, K., Combs, D. J., and Sehgal, A. (2011). Post-translational regulation and nuclear entry of TIMELESS and PERIOD are affected in new timeless mutant. *J. Neurosci.* 31, 9982–9990. doi: 10.1523/JNEUROSCI.0993-11.2011

Hardin, P. E. (2011). Molecular genetic analysis of circadian timekeeping in Drosophila. *Genet. Circadian Rhythms* 74, 141–173. doi: 10.1016/B978-0-12-387690-4.00005-2

Hardin, P. E., Hall, J. C., and Rosbash, M. (1990). Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. *Nature* 343, 536–540. doi: 10.1038/343536a0

Hardin, P. E., Hall, J. C., and Rosbash, M. (1992). Circadian oscillations in period gene messenger-RNA levels are transcriptionally regulated. *Proc. Natl. Acad. Sci. U.S.A.* 89, 11711–11715. doi: 10.1073/pnas.89.24.11711

Hastings, J. W., and Sweeney, B. M. (1957). On the mechanism of temperature independence in a biological clock. *Proc. Natl. Acad. Sci. U.S.A.* 43, 804–811. doi: 10.1073/pnas.43.9.804

Izumo, M., Johnson, C. H., and Yamazaki, S. (2003). Circadian gene expression in mammalian fibroblasts revealed by real-time luminescence reporting: temperature compensation and damping. *Proc. Natl. Acad. Sci. U.S.A.* 100, 16089–16094. doi: 10.1073/pnas.2536313100

Jang, A. R., Moravcevic, K., Saez, L., Young, M. W., and Sehgal, A. (2015). Drosophila TIM binds importin alpha1, and acts as an adapter to transport PER to the nucleus. *PLoS Genet.* 11:e1004974. doi: 10.1371/journal.pgen.1004974

Kamae, Y., and Tomioka, K. (2012). timeless is an essential component of the circadian clock in a primitive insect, the firebrat Thermobia domestica. *J. Biol. Rhythms* 27, 126–134. doi: 10.1177/0748730411435997

Kaushik, R., Nawathean, P., Busza, A., Murad, A., Emery, P., and Rosbash, M. (2007). PER-TIM interactions with the photoreceptor cryptochrome mediate circadian temperature responses in Drosophila. *PLoS Biol.* 5:e0050146. doi: 10.1371/journal.pbio.0050146

Ko, H. W., Kim, E. Y., Chiu, J., Vanselow, J. T., Kramer, A., and Edery, I. (2010). A hierarchical phosphorylation cascade that regulates the timing of PERIOD nuclear entry reveals novel roles for proline-directed kinases and GSK-3 beta/SGG in circadian clocks. *J. Neurosci.* 30, 12664–12675. doi: 10.1523/Jneurosci.1586-10.2010

Kobelkova, A., Bajgar, A., and Dolezel, D. (2010). Functional molecular analysis of a circadian clock Gene timeless promoter from the drosophilid fly *Chymomyza costata*. *J. Biol. Rhythm* 25, 399–409. doi: 10.1177/0748730410385283

Kobelkova, A., Zavodska, R., Sauman, I., Bazalova, O., and Dolezel, D. (2015). Expression of clock genes period and timeless in the central nervous system of the Mediterranean flour moth, *Ephestia kuehniella*. *J. Biol. Rhythms* 30, 104–116. doi: 10.1177/0748730414568430

Kondo, S., and Ueda, R. (2013). Highly improved gene targeting by germline-specific Cas9 expression in Drosophila. *Genetics* 195, 715–721. doi: 10.1534/genetics.113.156737

Konopka, R. J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proc. Natl. Acad. Sci. U.S.A.* 68, 2112–2116. doi: 10.1073/pnas.68.9.2112

Konopka, R. J., Hamblencoyle, M. J., Jamison, C. F., and Hall, J. C. (1994). An ultrashort clock mutation at the period locus of Drosophila melanogaster that reveals some new features of the flys circadian system. *J. Biol. Rhythm* 9, 189–216. doi: 10.1177/074873049400900303

Konopka, R. J., Pittendrigh, C., and Orr, D. (1989). Reciprocal behaviour associated with altered homeostasis and photosensitivity of Drosophila clock mutants. *J. Neurogenet.* 6, 1–10. doi: 10.3109/01677068909107096

Kosugi, S., Hasebe, M., Tomita, M., and Yanagawa, H. (2008). Nuclear export signal consensus sequences defined using a localization-based yeast selection system. *Traffic* 9, 2053–2062. doi: 10.1111/j.1600-0854.2008.00825.x

Kotwica-Rolinska, J., Chodakova, L., Chvalova, D., Kristofova, L., Fenclova, I., Provaznik, J., et al. (2019). CRISPR/Cas9 genome editing introduction and optimization in the non-model insect *Pyrrhocoris apterus*. *Front. Physiol.* 10:891. doi: 10.3389/fphys.2019.00891

Kotwica-Rolinska, J., Pivarciova, L., Vaneckova, H., and Dolezel, D. (2017). The role of circadian clock genes in the photoperiodic timer of the linden bug, *Pyrrhocoris apterus*, during the nymphal stage. *Physiol. Entomol.* 42, 266–273. doi: 10.1111/phen.12197

Landskron, J., Chen, K. F., Wolf, E., and Stanewsky, R. (2009). A role for the PERIOD:PERIOD homodimer in the Drosophila circadian clock. *PLoS Biol.* 7:e1000003. doi: 10.1371/journal.pbio.1000003

Levine, J. D., Funes, P., Dowse, H. B., and Hall, J. C. (2002). Signal analysis of behavioral and molecular cycles. *BMC Neurosci.* 3:1. doi: 10.1186/1471-2202-3-1

Li, Y. H., Liu, X., Vanselow, J. T., Zheng, H., Schlosser, A., and Chiu, J. C. (2019). O-GlcNAcylation of PERIOD regulates its interaction with CLOCK and timing of circadian transcriptional repression. *PLoS Genet.* 15:e1007953. doi: 10.1371/journal.pgen.1007953

Majercak, J., Sidote, D., Hardin, P. E., and Edery, I. (1999). How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron* 24, 219–230. doi: 10.1016/s0896-6273(00)80834-x

Martinek, S., Inonog, S., Manoukian, A. S., and Young, M. W. (2001). A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. *Cell* 105, 769–779. doi: 10.1016/S0092-8674(01)00383-X

Matsumoto, A., Tomioka, K., Chiba, Y., and Tanimura, T. (1999). timrit lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. *Mol. Cell. Biol.* 19, 4343–4354. doi: 10.1128/mcb.19.6.4343

Meyer, P., Saez, L., and Young, M. W. (2006). PER-TIM interactions in living Drosophila cells: an interval timer for the circadian clock. *Science* 311, 226–229. doi: 10.1126/science.1118126

Montelli, S., Mazzotta, G., Vanin, S., Caccin, L., Corra, S., De Pitta, C., et al. (2015). period and timeless mRNA splicing profiles under natural conditions in Drosophila melanogaster. *J. Biol. Rhythms* 30, 217–227. doi: 10.1177/0748730415583575

Nakajima, M., Imai, K., Ito, H., Nishiwaki, T., Murayama, Y., Iwasaki, H., et al. (2005). Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* 308, 414–415. doi: 10.1126/science.1108451

Nawathean, P., and Rosbash, M. (2004). The doubletime and CKII kinases collaborate to potentiate Drosophila PER transcriptional repressor activity. *Mol. Cell.* 13, 213–223. doi: 10.1016/S1097-2765(03)00503-3

Ozkaya, O., and Rosato, E. (2012). The circadian clock of the fly: a neurogenetics journey through time. *Adv. Genet.* 77, 79–123. doi: 10.1016/B978-0-12-387687-4.00004-0

Pittendrigh, C. S. (1954). On temperature independence in the clock system controlling emergence time in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 40, 1018–1029. doi: 10.1073/pnas.40.10.1018

Pivarciova, L., Vaneckova, H., Provaznik, J., Wu, B. C., Pivarci, M., Peckova, O., et al. (2016). Unexpected geographic variability of the free running period in the linden bug, *Pyrrhocoris apterus*. *J. Biol. Rhythms* 31, 568–576. doi: 10.1177/0748730416671213

Port, F., Chen, H. M., Lee, T., and Bullock, S. L. (2014). Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2967–E2976. doi: 10.1073/pnas.1405500111

Poupardin, R., Schottner, K., Korbelova, J., Provaznik, J., Dolezel, D., Pavlinic, D., et al. (2015). Early transcriptional events linked to induction of diapause revealed by RNAseq in larvae of drosophilid fly, *Chymomyza costata*. *BMC Genomics* 16:720. doi: 10.1186/s12864-015-1907-4

Price, J. L. (2005). Genetic screens for clock mutants in Drosophila. *Method Enzymol.* 393, 35–60. doi: 10.1016/S0076-6879(05)93003-6

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

Ren, X., Yang, Z., Xu, J., Sun, J., Mao, D., Hu, Y., et al. (2014). Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in Drosophila. *Cell Rep.* 9, 1151–1162. doi: 10.1016/j.celrep.2014.09.044

Rothenfluh, A., Abodeely, M., Price, J. L., and Young, M. W. (2000a). Isolation and analysis of six timeless alleles that cause short- or long-period circadian rhythms in Drosophila. *Genetics* 156, 665–675.

Rothenfluh, A., Young, M. W., and Saez, L. (2000b). A TIMELESS-independent function for PERIOD proteins in the Drosophila clock. *Neuron* 26, 505–514. doi: 10.1016/S0896-6273(00)81182-4

Ruoff, P. (1992). Introducing temperature compensation in any reaction kinetic oscillator model. *J. Interdiscipl. Cycle* 23, 92–99.

Rutila, J. E., Zeng, H., Le, M., Curtin, K. D., Hall, J. C., and Rosbash, M. (1996). The timSL mutant of the Drosophila rhythm gene timeless manifests allele-specific interactions with period gene mutants. *Neuron* 17, 921–929. doi: 10.1016/s0896-6273(00)80223-8

Saez, L., Derasmo, M., Meyer, P., Stieglitz, J., and Young, M. W. (2011). A key temporal delay in the circadian cycle of Drosophila is mediated by a nuclear localization signal in the timeless protein. *Genetics* 188, 591–U166. doi: 10.1534/genetics.111.127225

Saez, L., and Young, M. W. (1996). Regulation of nuclear entry of the Drosophila clock proteins period and timeless. *Neuron* 17, 911–920. doi: 10.1016/S0896-6273(00)80222-6

Sathyanarayanan, S., Zheng, X., Xiao, R., and Sehgal, A. (2004). Posttranslational regulation of Drosophila PERIOD protein by protein phosphatase 2A. *Cell* 116, 603–615. doi: 10.1016/s0092-8674(04)00128-x

Schmid, B., Helfrich-Forster, C., and Yoshii, T. (2011). A new ImageJ plug-in "ActogramJ" for chronobiological analyses. *J. Biol. Rhythms* 26, 464–467. doi: 10.1177/0748730411414264

Sehadova, H., Glaser, F. T., Gentile, C., Simoni, A., Giesecke, A., Albert, J. T., et al. (2009). Temperature entrainment of Drosophila's circadian clock involves the gene nocte and signaling from peripheral sensory tissues to the brain. *Neuron* 64, 251–266. doi: 10.1016/j.neuron.2009.08.026

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

Shafer, O. T., Rosbash, M., and Truman, J. W. (2002). Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of Drosophila melanogaster. *J. Neurosci.* 22, 5946–5954.

Shinohara, Y., Koyama, Y. M., Ukai-Tadenuma, M., Hirokawa, T., Kikuchi, M., Yamada, R. G., et al. (2017). Temperature-sensitive substrate and product binding underlie temperature-compensated phosphorylation in the clock. *Mol. Cell.* 67, 783–798. doi: 10.1016/j.molcel.2017.08.009

Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M., and Hall, J. C. (1988). Antibodies to the period gene product of Drosophila reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* 1, 141–150. doi: 10.1016/0896-6273(88)90198-5

Stanewsky, R., Frisch, B., Brandes, C., HamblenCoyle, M. J., Rosbash, M., and Hall, J. C. (1997). Temporal and spatial expression patterns of transgenes containing increasing amounts of the Drosophila clock gene period and a lacZ reporter: mapping elements of the PER protein involved in circadian cycling. *J. Neurosci.* 17, 676–696.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S. A., et al. (1998). The cry(b) mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. *Cell* 95, 681–692. doi: 10.1016/S0092-8674(00)81638-4

Tataroglu, O., and Emery, P. (2015). The molecular ticks of the Drosophila circadian clock. *Curr. Opin. Insect. Sci.* 7, 51–57. doi: 10.1016/j.cois.2015.01.002

Tauber, E., Zordan, M., Sandrelli, F., Pegoraro, M., Osterwalder, N., Breda, C., et al. (2007). Natural selection favors a newly derived timeless allele in Drosophila melanogaster. *Science* 316, 1895–1898. doi: 10.1126/science.1138412

Tomioka, K., and Matsumoto, A. (2015). Circadian molecular clockworks in non-model insects. *Curr. Opin. Insect. Sci.* 7, 58–64. doi: 10.1016/j.cois.2014.12.006

Urbanova, V., Bazalova, O., Vaneckova, H., and Dolezel, D. (2016). Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. *Insect Biochem. Mol. Biol.* 70, 184–190. doi: 10.1016/j.ibmb.2016.01.003

Wülbeck, C., Szabo, G., Shafer, O. T., Helfrich-Forster, C., and Stanewsky, R. (2005). The novel Drosophila tim(blind) mutation affects behavioral rhythms but not periodic eclosion. *Genetics* 169, 751–766. doi: 10.1534/genetics.104.036244

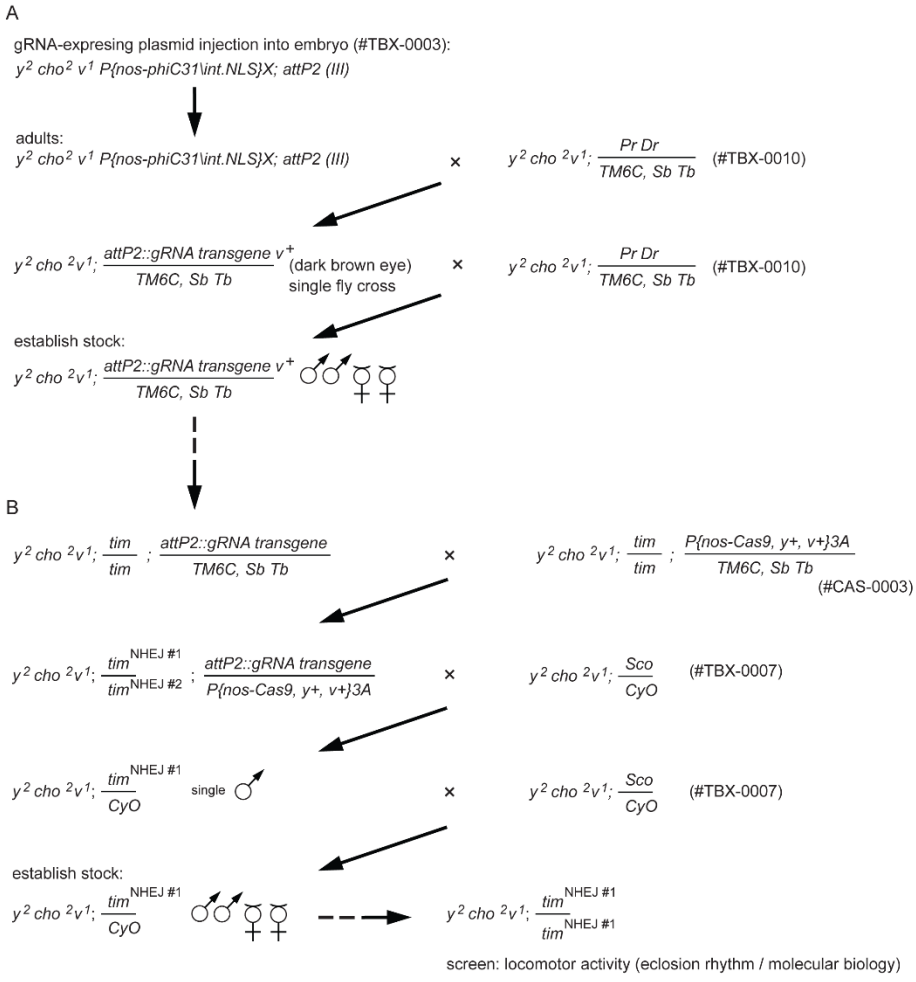
Zhang, Z., Cao, W., and Edery, I. (2018). The SR protein B52/SRp55 regulates splicing of the period thermosensitive intron and mid-day siesta in Drosophila. *Sci. Rep.* 8:1872. doi: 10.1038/s41598-017-18167-3

Zhou, M., Kim, J. K., Eng, G. W., Forger, D. B., and Virshup, D. M. (2015). A Period2 phosphoswitch regulates and temperature compensates circadian period. *Mol. Cell.* 60, 77–88. doi: 10.1016/j.molcel.2015.08.022

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

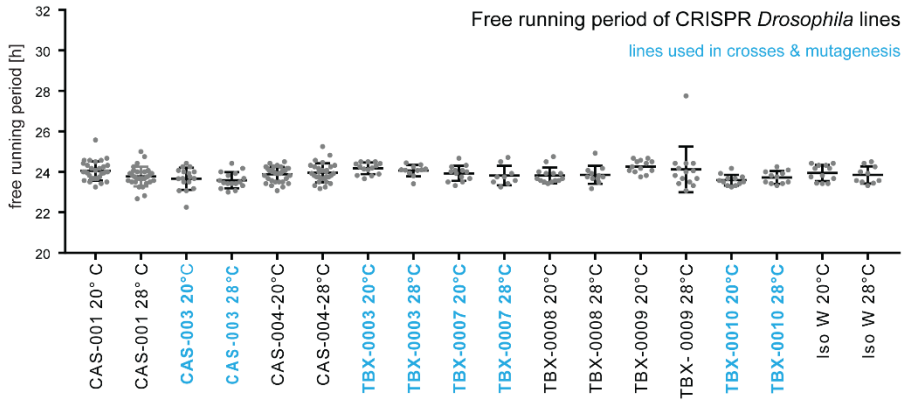
# Supplementary material

**Supplementary Figures S1**| Scheme of genetic crosses used to (**A**) obtain gRNA-expressing line, and (**B**) to initiate NHEJ by combining gRNA-expressing line with CAS9-expressing line. The 'TBX' number correspond to Kondo and Ueda (2013).



**Supplementary Figures S2**|(**A**)Free running period and (**B**) detailed genotypes of lines used in the current study (names in blue in panel A) and lines that might be used for similar experiments.

A

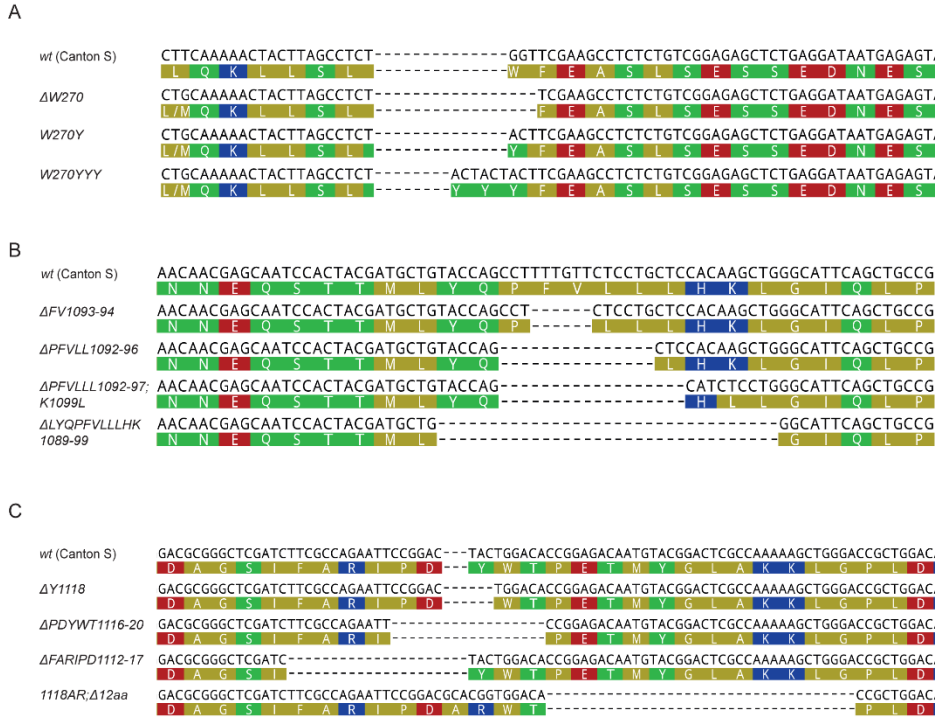



B

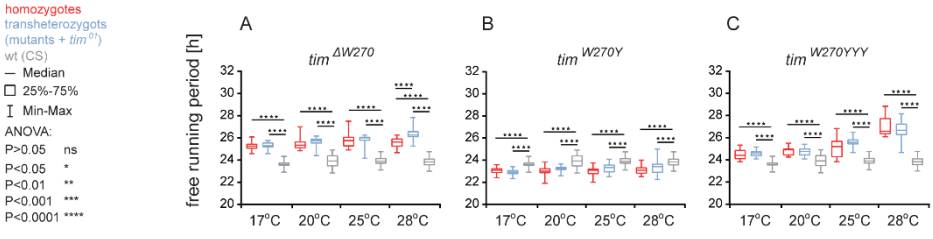
Genotypes of *Drosophila* lines used in mutagenesis and lines suitable for similar experiments (Kondo and Ueda 2013; https://shigen.nig.ac.jp/fly/nigfly/cas9/):

| | |
|---|---|
| CAS-0001 | y2 cho2 v1; attP40{nos-Cas9}/CyO |
| CAS-0003 | y2 cho2 v1; P{nos-Cas9, y+, v+}3A/TM6C, Sb Tb |
| CAS-0004 | y2 cho2 v1; Sp/CyO, P{nos-Cas9, y+, v+}2A |
| TBX-0007 | y2 cho2 v1; Sco/CyO |
| TBX-0008 | y2 cho2 v1/Yhs-hid; Sp/CyO |
| TBX-0008 | y2 cho2 v1; Sp hs-hid/CyO |
| TBX-0010 | y2 cho2 v1; Pr Dr/TM6C, Sb Tb |

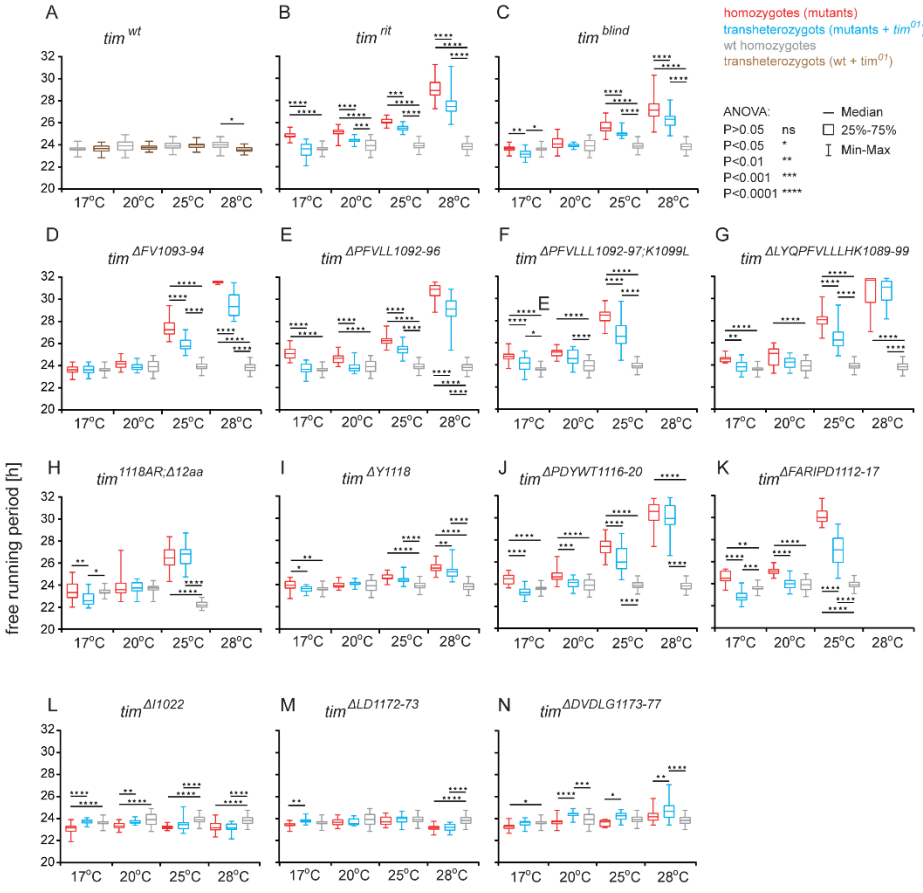
**Supplementary Figures S3| (A, B, C)** DNA and protein sequences of obtained mutants compared to control (*wt*, *wild type*)



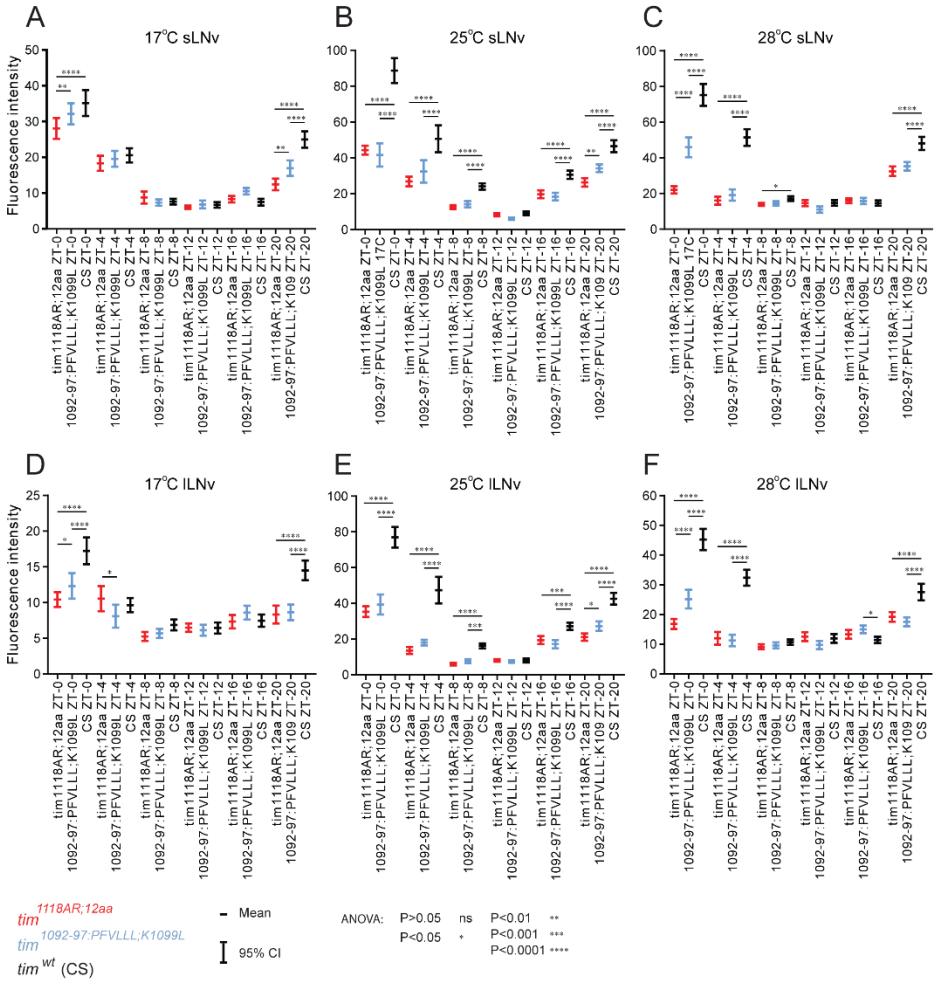

**Supplementary Figures S4**| Statistical comparison of the free running periods for mutants near the *ultralong* region of *timeless*.



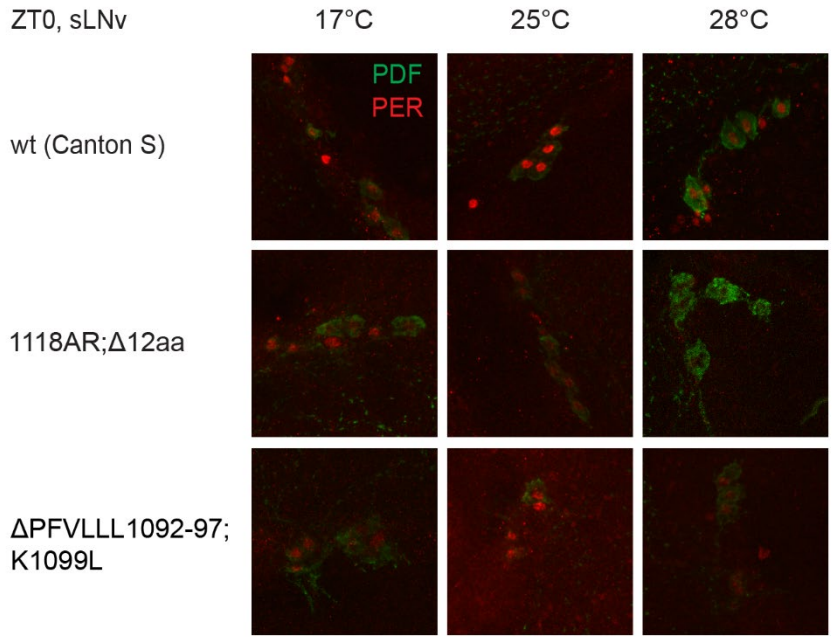
**Supplementary Figures S5**| Statistical comparison of the free running periods for mutants near the *blind* and *ritsu* regions of *timeless.*



**Supplementary Figures S6**| Statistical comparison of the PER staining intensity between three mutants at three temperatures.



**Supplementary Figures S7**| Examples of PER stainings at ZT0 (=light on) at three temperatures.



| Table S1. part A | Free-running locomotion in DD at different temperatures | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 18°C | | | 25°C | | | 28°C | | |
| genotype | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) |
| $tim^{blind};cry^{01}$ | 51 (84) | 24 (+1/-1) | 10 (16) | 54 (96) | 25 (+0.5/-0.5) | 2 (4) | 59 (98) | 26 (+1/-1.5) | 1 (2) |
| $tim^{blind};cry^{02}$ | 26 (87) | 24 (+1/-1) | 4 (13) | 25 (96) | 25 (+1/-1) | 1 (4) | 31 (97) | 26 (+2/-0.5) | 1 (3) |
| $tim^{blind};cry^{03}$ | 28 (93) | 24 (+1/-1) | 2 (7) | 28 (97) | 25.5 (+1/-0.5) | 1 (3) | 28 (97) | 26.5 (+2/-1.5) | 1 (3) |
| $tim^{blind};cry^{b}$ | 20 (87) | 24 (+1.5/-0.5) | 3 (13) | 26 (100) | 25.5 (+1/-1.5) | 0 (0) | 31 (100) | 26.5 (+1.5/-1) | 0 (0) |
| $tim^{blind};cry^{m}$ | 16 (80) | 24 (+2.5/-1) | 4 (20) | 29 (94) | 26 (+0.5/-0.5) | 2 (6) | 28 (93) | 27 (+1.5/-0.5) | 2 (7) |
| $tim^{blind};Sb/TM6B$ | 21 (95) | 23.5 (+1/-1) | 1 (5) | 29 (100) | 25 (+1.5/-0.5) | 0 (0) | 24 (100) | 26 (+2/-0) | 0 (0) |
| $tim^{rit};cry^{01}$ | 23 (100) | 25 (+1/-1) | 0 (0) | 26 (93) | 26.5 (+1.5/-1.5) | 2 (7) | 18 (86) | 28 (+2.5/-2) | 3 (14) |
| $tim^{rit};cry^{02}$ | 23 (100) | 24.5 (+1/-1) | 0 (0) | 28 (93) | 26.5 (+2/-1.5) | 2 (7) | 19 (83) | 30 (+3.5/-3.5) | 4 (7) |
| $tim^{rit};cry^{03}$ | 24 (100) | 25 (+1/-2) | 0 (0) | 25 (86) | 28 (+1.5/-2.5) | 4 (14) | 14 (88) | 31.5 (+2/-2.5) | 2 (12) |
| $tim^{rit};cry^{b}$ | 22 (96) | 24.5 (+0.5/-1) | 1 (4) | 26 (96) | 26.5 (+3/-1) | 1 (4) | 17 (85) | 31 (+3.5/-3) | 3 (15) |
| $tim^{rit};cry^{m}$ | 21 (100) | 24.5 (+0.5/-0.5) | 0 (0) | 31 (100) | 27.5 (+1.5/-1.5) | 0 (0) | 12 (63) | 30 (+3.5/-3.5) | 7 (37) |
| $tim^{rit};Sb/TM6B$ | 16 (100) | 25 (+0.5/-0.5) | 0 (0) | 31 (100) | 26.5 (+1.5/-1.5) | 0 (0) | 15 (94) | 28.5 (+2.5/-0.5) | 1 (6) |
| $tim^{S1};cry^{01}$ | 7 (30) | 20.5 (+0.5/-0.7) | 16 (70) | 18 (64) | 20 (+1/-1.5) | 10 (36) | 16 (70) | 19.5 (+2.5/-1) | 7 (30) |
| $tim^{S1};cry^{02}$ | 3 (14) | 21 (+1.5/-0.5) | 18 (86) | 13 (54) | 20.5 (+1/-0.8) | 11 (46) | 14 (70) | 19.7 (+0.3/-0.7) | 6 (30) |
| $tim^{S1};cry^{03}$ | 18 (75) | 21.5 (+0.5/-1.5) | 6 (25) | 25 (86) | 21.5 (+1/-2) | 4 (14) | 18 (100) | 20 (+1/-2) | 0 (0) |
| $tim^{S1};cry^{b}$ | 12 (52) | 20.5 (+1/-0.5) | 11 (48) | 22 (79) | 20 (+2.5/-1.5) | 6 (21) | 17 (89) | 19.7 (+1.8/-1.2) | 2 (11) |
| $tim^{S1};cry^{m}$ | 13 (57) | 21 (+0.5/-1) | 10 (43) | 21 (95) | 21 (+1/-1.5) | 1 (5) | 21 (91) | 21 (+0.5/-1) | 2 (9) |
| $tim^{S1};Sb/TM6B$ | 18 (78) | 21 (+1/-2) | 5 (22) | 26 (97) | 21 (+1/-2) | 2 (3) | 16 (100) | 20.5 (+1/-0.5) | 0 (0) |
| $tim^{L1};cry^{01}$ | 19 (83) | 26.5 (+1/-2) | 4 (17) | 11 (92) | 26 (+1/-0.5) | 1 (8) | 10 (100) | 25 (+2/-0.5) | 0 (0) |
| $tim^{L1};cry^{02}$ | 9 (64) | 27 (+1/-1) | 5 (36) | 5 (83) | 27 (+0.5/-1) | 1 (17) | 10 (100) | 26 (+1/-0.5) | 0 (0) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $tim^{L1};cry^{03}$ | 20 (91) | 26 (+1/-0.5) | 2 (9) | 17 (89) | 27 (+0.5/-1) | 2 (11) | 16 (80) | 26 (+1/-0.5) | 4 (20) |
| $tim^{L1};cry^{b}$ | 6 (86) | 26.5 (+0.5/-1) | 1 (14) | 1 | | | 11 (100) | 25.5 (+0.5/-1.5) | 0 (0) |
| $tim^{L1};cry^{m}$ | 10 (77) | 26.25 (+0.25/-1.25) | 3 (23) | 24 (100) | 27.5 (+1/-2) | 0 (0) | 21 (100) | 26 (+1/-0) | 0 (0) |
| $tim^{L1};Sb/TM6B$ | 7 (100) | 25 (+1/-0.5) | 0 (0) | 11 (85) | 26 (+1/-1.5) | 2 (15) | 8 (100) | 25.75 (+0.25/-0.25) | 0 (0) |

**Table S1. part B** **Free-running locomotion in DD at different temperatures**

| Genotype | 18°C | | | 25°C | | | 28°C | | |
|---|---|---|---|---|---|---|---|---|---|
| | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) |
| $tim^{UL};cry^{01}$ | 16 (59) | 32 (+1.5/-3.5) | 11 (41) | 6 (25) | 33.25 (+2.25/-2.75) | 18 (75) | 12 (44) | 33 (+1.5/-0.5) | 15 (56) |
| $tim^{UL};cry^{02}$ | 14 (47) | 32 (+1.5/-3.5) | 16 (53) | 7 (32) | 35 (+0.5/-2) | 15 (78) | 11 (55) | 34.5 (+1/-1) | 9 (45) |
| $tim^{UL};cry^{03}$ | 15 (63) | 33 (+2/-3.5) | 9 (37) | 9 (41) | 34 (+1.5/-1) | 13 (59) | 9 (43) | 34 (+1/-1.5) | 12 (57) |
| $tim^{UL};cry^{b}$ | 22 (79) | 31.5 (+1.5/-2) | 6 (21) | 11 (69) | 33 (+1/-0.5) | 7 (31) | 17 (65) | 33.5 (+1/-1) | 9 (35) |
| $tim^{UL};cry^{m}$ | 35 (81) | 30 (+1.5/-1.5) | 8 (19) | 39 (72) | 32 (+2/-3) | 15 (28) | 50 (85) | 32.5 (+1.5/-1.5) | 9 (15) |
| $tim^{UL};Sb/TM6B$ | 16 (100) | 30.75 (+2.25/-1.75) | 0 (0) | 15 (75) | 30 (+2.5/-2) | 5 (25) | 13 (62) | 33 (+1/-0.5) | 8 (38) |
| $per^{SLIH};Sp/CyO;\ cry^{01}$ | 21 (95) | 28 (+1/-2) | 1 (5) | 20 (83) | 27 (+1./-1) | 4 (17) | 10 (71) | 25.5 (+1/-0.5) | 4 (28) |
| $per^{SLIH};Sp/CyO;\ cry^{02}$ | 21 (91) | 27.5 (+1.5/-1) | 2 (9) | 24 (89) | 27 (+1.5/-1) | 3 (11) | 14 (70) | 26 (+1/-0.5) | 6 (30) |
| $per^{SLIH};Sp/CyO;\ cry^{03}$ | 21 (95) | 28.5 (+1/-2) | 1 (5) | 23 (92) | 28 (+1.5/-1.2) | 2 (8) | 19 (95) | 27 (+0.5/-1) | 1 (5) |
| $per^{SLIH};Sp/CyO;\ cry^{b}$ | 21 (95) | 28 (+1/-0.5) | 1 (5) | 20 (91) | 27 (+0.5./-1) | 2 (9) | 17 (100) | 26 (+0.5/-0) | 0 (0) |
| $per^{SLIH};Sp/CyO;\ cry^{m}$ | 23 (100) | 28.5 (+1/-1) | 0 (0) | 29 (97) | 28 (+0.5/-1) | 1 (3) | 22 (100) | 26.5 (+1.5/-0.5) | 0 (0) |
| $per^{SLIH};Sp/CyO;\ Sb/TM6B$ | 20 (95) | 28 (+1/-1.5) | 1 (5) | 24 (92) | 27.5 (+0.5/-1) | 2 (8) | 14 (100) | 26 (+1.5/-0) | 0 (0) |
| $per^{T};Sp/CyO;\ cry^{01}$ | 16 (73) | 16.5 (+0.5/-0.5) | 6 (27) | 19 (66) | 16 (+1/-0.5) | 10 (34) | 6 (38) | 15.5 (+0.8/-0.5) | 10 (62) |
| $per^{T};Sp/CyO;\ cry^{02}$ | 17 (74) | 16.3 (+0.7/-0.8) | 6 (26) | 10 (71) | 16.15 (+0.85/-0.85) | 4 (29) | 4 (44) | 15.5 (+1.15/-0.35) | 5 (56) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *per$^{T}$;Sp/CyO; cry$^{03}$* | 19 (83) | 16 (+1/-0.3) | 4 (17) | 17 (94) | 16.3 (+0.7/-0.8) | 1 (6) | 18 (86) | 16 (+0.7/-1) | 3 (6) |
| *per$^{T}$;Sp/CyO; cry$^{b}$* | 12 (67) | 16.5 (+0.7/-1) | 6 (33) | 9 (82) | 16.5 (+0.8/-0.5) | 2 (18) | 8 (62) | 17.15 (+0.35/-0.65) | 5 (38) |
| *per$^{T}$;Sp/CyO; cry$^{m}$* | 15 (79) | 16.5 (+0.5/-1.3) | 4 (21) | 21 (91) | 16.5 (+1/-0.5) | 2 (9) | 14 (93) | 16.4 (+0.9/-0.4) | 1 (7) |
| *per$^{T}$;Sp/CyO; Sb/TM6B* | 15 (75) | 16.5 (+0.5/-1) | 5 (25) | 17 (100) | 15.5 (+0.8/-0.5) | 0 (0) | 11 (85) | 15.7 (+1.3/-0.7) | 2 (15) |

**Table S1. part C** **Free-running locomotion in DD at different temperatures**

| Genotype | 18°C | | | 25°C | | | 28°C | | |
|---|---|---|---|---|---|---|---|---|---|
| | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) |
| *y per$^{T}$;Sp/CyO; cry$^{01}$* | 20 (83) | 16.5 (+0.5/-0.5) | 4 (17) | 12 (67) | 16.15 (+1.15/-0.65) | 6 (33) | 11 (65) | 16 (+2/-1) | 6 (35) |
| *y per$^{T}$;Sp/CyO; cry$^{02}$* | 4 (18) | 16.85 (+0.65/-0.35) | 18 (81) | 11 (46) | 15.7 (+1.3/-0.4) | 13 (54) | 4 (29) | 16.75 (+0.3/-0.75) | 10 (71) |
| *y per$^{T}$;Sp/CyO; cry$^{03}$* | 11 (50) | 16.5 (+1/-0.8) | 11 (50) | 25 (86) | 16.7 (+1.3/-1.2) | 4 (14) | 10 (45) | 16 (+0.5/-1.5) | 12 (55) |
| *y per$^{T}$;Sp/CyO; cry$^{b}$* | 15 (65) | 16.3 (+1.2/-1) | 8 (35) | 19 (68) | 16.3 (+0.7/-1.3) | 9 (32) | 11 (55) | 16.5 (+1.5/-1.2) | 9 (45) |
| *y per$^{T}$;Sp/CyO; cry$^{m}$* | 16 (70) | 16.7 (+0.6/-1.4) | 7 (30) | 19 (90) | 16.7 (+0.3/-0.4) | 2 (10) | 23 (100) | 16.3 (+1/-0.6) | 0 (0) |
| *y per$^{T}$;Sp/CyO; Sb/TM6B* | 16 (76) | 16.5 (+0.5/-1) | 5 (24) | 19 (76) | 16 (+1/-1) | 6 (24) | 7 (78) | 16.5 (+1/-0.5) | 2 (22) |
| *per$^{S}$;Sp/CyO; cry$^{01}$* | 21 (84) | 20 (+0.5/-1) | 4 (16) | 14 (67) | 19.15 (+1.35/-0.65) | 7 (33) | 13 (93) | 18.7 (+0.6/-0.4) | 1 (7) |
| *per$^{S}$;Sp/CyO; cry$^{02}$* | 5 (25) | 19 (+0.3/0.5) | 15 (75) | 12 (57) | 19.25 (+1.25/-1.25) | 9 (43) | 13 (72) | 18.85 (+0.65/-0.85) | 5 (28) |
| *per$^{S}$;Sp/CyO; cry$^{03}$* | 12 (60) | 19.5 (+0.5/-0.5) | 8 (40) | 19 (86) | 19 (+2/-0.3) | 3 (14) | 13 (87) | 18.8 (+0.9/-0.8) | 2 (13) |
| *per$^{S}$;Sp/CyO; cry$^{b}$* | 12 (67) | 19.5 (+0.5/-1) | 6 (33) | 2 | | | 6 | 18.7 (+1/0.4) | 0 |
| *per$^{S}$;Sp/CyO; cry$^{m}$* | 21 (91) | 19.5 (+1.5/-1.2) | 2 (9) | 25 (100) | 19.3 (+0.7/-0.3) | 0 (0) | 21 (100) | 19 (+0.7/-1) | 0 (0) |
| *per$^{S}$;Sp/CyO; Sb/TM6B* | 13 (93) | 19.8 (+0.2/-1.1) | 1 (7) | 18 (86) | 18.5 (+0.5/-0.5) | 3 (14) | 15 (94) | 18.5 (+0.5/-0.5) | 1 (6) |

| Table S1. part D | Free-running locomotion in DD at different temperatures | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Genotype | 18°C | | | 25°C | | | 28°C | | |
| | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) | *N* rhythmic (%) | FRP (+/-) [hrs] | *N* non-rhythmic (%) |
| *per$^{L}$; +/+; cry$^{01}$* | 5 | 27 (+0.5/-0.5) | 0 | 6 (38) | 29.5 (+0.5/-0.5) | 10 (62) | 5 | 29.5 (+0.5/-0.5) | 0 |
| *per$^{L}$; +/+; cry$^{02}$* | 20 (83) | 27.5 (+0.5/-1.5) | 4 (17) | 17 (77) | 28 (+1/-0.5) | 5 (23) | 17 (89) | 29 (+0.5/-0.5) | 2 (11) |
| *per$^{L}$; +/+; cry$^{03}$* | 22 (92) | 26.5 (+1.0/-0.5) | 2 (8) | 17 (74) | 29.5 (+0.5/-2) | 6 (26) | 17 (81) | 30 (+0.5/-0.5) | 4 (19) |
| *per$^{L}$; +/+; cry$^{b}$* | 11 (79) | 27 (+0.5/-1.5) | 3 (21) | 14 (88) | 29 (+1/-0.5) | 2 (12) | 9 (82) | 29 (+1.5/-0.5) | 2 (18) |
| *per$^{L}$; +/+; cry$^{m}$* | 16 (76) | 28 (+1/-1) | 5 (24) | 18 (75) | 30 (+2/-2.5) | 6 (25) | 14 (82) | 32 (+1/-1) | 3 (18) |
| *per$^{L}$; +/+; Sb/TM6B* | 14 (70) | 27.25 (+0.75/-0.75) | 6 (30) | 15 (79) | 30.5 (+2.5/-2) | 4 (21) | 10 (100) | 30 (+1.5/-1) | 0 (0) |
| *Sp/Cyo;cry$^{01}$* | 22 (100) | 23.5 (+1/-0.5) | 0 (0) | 20 (100) | 23.5 (+1/-0.5) | 0 (0) | 13 (100) | 23 (+1.5/-0) | 0 |
| *Sp/Cyo;cry$^{02}$* | 16 (84) | 23.5 (+2/-0.5) | 3 (15) | 18 (100) | 23.75 (+0.75/-1.25) | 0 (0) | 13 (93) | 23 (+1.5/-0) | 1 (7) |
| *Sp/CyO;cry$^{03}$* | 23 (100) | 24 (+0.5/-0.5) | 0 (0) | 24 (100) | 24 (+0.5/-0.5) | 0 (0) | 14 (100) | 23.5 (+0.5/-0.5) | 0 (0) |
| *Sp/CyO;cry$^{b}$* | 12 (100) | 23.5 (+1/-0.5) | 0 | 9 (90) | 24 (+0.5/-1) | 1 (10) | 9 | 23.5 (+0.5/-0.5) | 0 |
| *Sp/CyO;cry$^{m}$* | 21 (95) | 23.5 (+1/-0.5) | 1 (5) | 19 (100) | 24 (+0.5/-0.5) | 0 (0) | 12 (86) | 23.5 (+0.5/-0) | 2 (14) |

# Chapter 2

Temperature dependent splicing in the regulation of circadian rhythms in *Drosophila melanogaster*

**Singh S**, Doležel D.

(Unpublished results)

In the **first chapter** we identified the regions in *timeless* (*tim)* critical for temperature compensation which shows altered temperature compensated phenotype once modified. Also, we showed that those regions are quite conserved across species and our study confirmed the importance of those regions for a functional circadian clock. Therefore, we further attempted to expand our understanding of the role of temperature in regulation of the expression of *tim*.

**In this chapter**, we focussed on a post-transcriptional mechanism, alternative splicing (AS), a regulatory step likely very important for the fine tuning of the clock. Since three recently published studies pointed to temperature-dependent alternative intron retention, we have decided to explore role of this intronic sequence by reverse genetics. Therefore, we sought to determine the functional significance of a thermo-sensitive intron retention event in *tim* which is known to happen at 25℃ and 28℃. Our study contributed to the understanding of alternative splicing in the regulation of circadian clock temperature compensation.

# Introduction

Splicing is an essential part of eukaryotic gene expression regulation process producing functional mRNA molecules by precisely regulated removal of introns from precursor messenger RNAs (mRNAs) followed by subsequent ligation of exons. The process of splicing which results in the combination of different exons from the same precursor mRNA is called alternative splicing (AS). It expands the coding capacity of genes and thus provides the basis for the impressive diversity of gene products originating from a substantially smaller set of genes (Brown et al., 2014; Nilsen & Graveley, 2010; Pan et al., 2008). Although, when a protein exists in different splicing variants, one splicing variant not only can exert its function in cooperation with the full-length protein (Fujita et al., 2009), or remarkably exert opposite functions compared to the full-length counterpart (Akgul et al., 2004). A classic example of AS is the sex determination pathway in *Drosophila* where proteins with completely different properties are produced (Bell et al., 1988). But AS may also be required to regulate the levels of the protein by post-transcriptional and/or post-translational regulation by miRNAs in a temperature dependent way (Martin Anduaga et al., 2019).

## Mechanism of splicing

Splicing is regulated by interactions between cellular splicing factors which are quite conserved between *Drosophila*, human and yeast (Mount & Salz, 2000) and RNA sequences in the pre-mRNA (Long & Caceres, 2009; Zheng, 2004). The whole mechanism is quite sensitive even to mild changes in the levels of splicing factors (Heinzen et al., 2008). Splicing reactions takes place in the nucleus of the cell and are

catalysed by spliceosomes, which are mainly composed of five small nuclear RNA (snRNA) molecules i.e. U1, U2, U4, U5 and U6, and around 100 associated auxiliary proteins to form a large enzymatic complex (Jurica & Moore, 2003). These five snRNAs interact with each other and together with a protein form small nuclear RiboNucleoProtein (snRNP) complexes which further assemble on the pre-mRNA to form the spliceosome and participates in the splicing reaction by interacting with intron consensus sequences containing GU at the 5' splice site (donor site) and AG at the 3' splice site (acceptor site). Among several proteins associated with the spliceosome, there are two highly conserved protein families: the splicing factors known as Ser/Arg-rich (SR) proteins and heterogeneous nuclear RiboNucleoProteins (hnRNPs) (Matlin et al., 2005). The SR proteins exhibit dual functionality in pre-mRNAs constitutive and alternative splicing. SR proteins are important to enhance suboptimal sites in a concentration-dependent manner by binding to exonic splicing enhancers (ESEs) and thereby forming a barrier to prevent exon skipping. In addition, SR proteins can also repress splicing when bound to splicing repressors or silencers (Kanopka et al., 1996; Pozzoli & Sironi, 2005). Mutations within ESEs can cause exon skipping showing the importance of these elements (Liu et al., 2001).

**Factors inducing alternative splicing**

Changes in gene expression determine how organisms grow and develop. Besides genes being turned off and on, many other processes regulate the expression and one of the most important mechanisms is AS, which generates more than one product from a gene.

Various studies suggested AS a response to stress due to pathological conditions (Dutertre et al., 2011), environmental conditions (Jakšić & Schlötterer, 2016; Kannan et al., 2018; Laloum et al., 2018; Tan et al., 2019), and others (Kucherenko & Shcherbata, 2018) where hundreds of genes undergo rapid changes in expression to help the organism to acclimatise to prevailing conditions. The circadian clock controls many diverse downstream processes and is itself affected by many environmental factors primarily light and temperature. Therefore, like other processes, AS is also known to play an important role in the expression of the core clock genes, *timeless* (*tim*) and *period* (*per*) (Boothroyd et al., 2007) thereby keep the clock in tune with the environment.

**Temperature dependent splicing in circadian clock genes**

Light and temperature are known to have a regulatory role on all physiological processes and so on circadian clock. Although light is a primary entraining signal, temperature has a more complex and interesting relationship to the clock. Goto and Denlinger in their study described a reciprocal correlation of temperature and the amount of *per* and *tim* mRNAs, where the abundance of *per* and *tim* mRNA fluctuates with change in temperature (Goto & Denlinger, 2002) which is further validated by Montelli and co-workers that the total amount of *tim* mRNA expressed is influenced by seasonal and even daily changes of temperature and day length in the natural conditions (Montelli et al., 2015). These changes in the transcript level primarily result from thermosensitive AS (Majercak et al., 1999; Martin Anduaga et al., 2019). Therefore, it is very likely that the alternatively spliced variants of central

circadian genes are required by organisms as an adaptive event to adjust to environmental changes.

Despite of changes in the environment the circadian clock is nicely regulated which is reflected in the expression of core clock genes that is tightly coupled during the light entrainment. This coupling breaks down in temperature entrainment, which leads to an advance in the expression of *per* and delay in the expression of *tim* but induces no overall differences at protein level (Boothroyd et al., 2007). There are several potential explanations on how the equal accumulation of PER and TIM proteins could be maintained regardless of the different expression levels. First, there could be a shift in the timing of the expression of both genes (Boothroyd et al., 2007) or the advance in the expression of *per* could be caused by thermosensitive splicing of this gene, which would then affect the expression levels of *tim* (Collins et al., 2004; Majercak et al., 1999). Finally, splicing occurring in both genes could explain the difference in their expression levels.

Studies in *D. melanogaster* have shown different thermosensitive spliced transcript variants in *tim* (Boothroyd et al., 2007; Martin Anduaga et al., 2019; Montelli et al., 2015; Shakhmantsir et al., 2018). Two isoforms of *tim*, *tim-cold* and *tim-short&cold*, have higher expression levels at low temperatures (18°C). The former results in a truncated protein lacking part of the cytoplasmic localization domain due to retention of the last intron, resulting into premature stop codon. The latter encodes a much smaller protein and in contrast to *tim-cold* is generated by an alternative cleavage and polyadenylation site located within the intron next to exon 9 of *tim*. Along with this, another isoform, *tim-tiny*

(referred as *tim-M* by Martin Anduaga et al. 2019) results from intron retention resulting into a premature stop codon at high temperatures (28°C) (Martin Anduaga et al., 2019; Shakhmantsir et al., 2018) (Fig. 1). The intron retained in *tim-tiny* isoform is just next to the exon 12, where several mutants with temperature compensation defect were identified (Singh et al., 2019; chapter 1). Therefore, we further explored the functional significance of *tim-tiny* isoform in the circadian clock by creating mutations within the intron as this isoform together with other isoforms regulates the levels of the canonical TIM in a temperature sensitive manner and may contribute to fine-tuning of PER and TIM protein oscillations with the daily thermal cycle ( Martin Anduaga et al., 2019; Shakhmantsir et al., 2018).

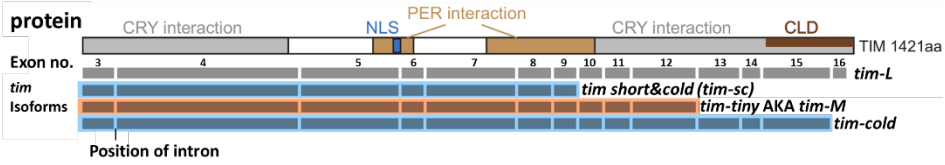



**Figure 1. Scheme of the alternatively spliced *tim* isoforms.** Schematic depiction of TIM protein with highlighted functional domains and interacting regions Below, schematic depiction of different *tim* isoforms separated by introns. In grey are constitutive exons (canonical isoform, *tim-L)*. The isoform shown in orange color is mainly found at high temperatures (*tim-tiny*), whereas the isoform shown in blue color are mainly found at low temperatures (*tim-sc* and *tim-cold*). On top of each exon corresponding numbers are mentioned to determine the exons absent in specific isoforms for comparison.

## Materials and methods

### Needle preparations

Eggs were injected by needles made of filamented borosilicate glass capillaries (outer diameter 1 mm, inner diameter 0.50 mm) (Sutter Instrument, Germany). Needles were pulled with the magnetic glass

microelectrode horizontal needle puller PN-31 (Narishige, Japan) using the following settings: temperature 78°C, magnet sub 20 and magnet main 100.

**Egg injections**

To collect eggs for injections around 500 flies were kept in egg laying chambers 1-2 days prior to the injection to get accustomed in a 40:60% male to female ratio. Eggs were collected in 20 minutes interval but before collecting the eggs for injections the plates were replaced every 20 minutes at least 4 times to allow flies to flush out older eggs withheld by females.

Eggs were injected with the FemtoJet system (Eppendorf) in the posterior end of the eggs (before the pole cells forms) aligned on a slide along the coverslip. After injections injected eggs were shifted into food vials supplied with moist paper-tissues and transferred to the incubator where they were kept until hatching (25°C). After 10-11 days, eclosed adults ($G_0$ adults) were used for genetic crosses.

**Genetic crosses**

Flies expressing Cas9 specifically in germ cells (nos-Cas9) from third chromosome insertion (NIG-FLY#: CAS-0003*; $y^2$ $cho^2$ $v^1$; P{nos-Cas9, y+, v+}3A/TM6C, Sb Tb*) were crossed with U6-gRNA-encoded transgenic strains (also located on the third chromosome). Resulting offspring thus expressed both gRNA and CAS9 on third chromosome, which potentially targeted *tim* gene located on the second chromosome and induce insertions and deletions as a result of non-homologous end joining (NHEJ) mechanism.

**Mosaicism in $G_0$ Generation**

For germline mosaicism testing, $G_0$ flies were allowed to mate with second chromosome balancer *$y^2$ cho$^2$ $v^1$; Sco/CyO* flies and after 2-3 days in mating gonads were dissected from those $G_0$ flies and DNA was extracted by squishing the gonads with the help of plastic pestle in 100 µl of the squishing buffer followed by PCR and heteroduplex mobility assay analysis as described below.

**Identification of mosaics by heteroduplex mobility assay**

From the PCR done on DNA extracted from $G_0$ flies five microliters of PCR reaction mixture was loaded onto 15% non-denaturing PAGE gels [15% acrylamide-bisacrylamide (29:1, w/w), 1X Tris-borate-EDTA (TBE), ammonium persulfate, and TEMED] and run in 0.5X TBE buffer for 2 h 20 min at 150 V in Mini PROTEAN Electrophoresis System (Bio-Rad), similar to the previously described protocol (Zhu et al., 2014). The gels were then stained for 10 min with GelRed nucleic acid stain solution in water (Biotium, United States) and imaged by Gel Documentation System Smart3-EZ (VWR, Belgium). The same setup is used for identification of mutants in F1 generation. The $G_0$ flies in which CRISPR/Cas9 resulted into indel results into the formation of heteroduplexes which runs slower on gel compared to corresponding homoduplexes.

**Identification of mutants**

The males and females of F1 generation of $G_0$ flies cross identified to be mosaics were further individually crossed with second chromosome balancer *$y^2$ cho$^2$ $v^1$; Sco/CyO* flies to establish lines with identically modified second chromosome (rest details mentioned in chapter 1). For

[redacted]

## References


Akgul, C., Moulding, D. A., & Edwards, S. W. (2004). Alternative splicing of Bcl-2-related genes: Functional consequences and potential therapeutic applications. *Cellular and Molecular Life Sciences*, 61, 2189-2199. https://doi.org/10.1007/s00018-004-4001-7

Bazalova, O., & Dolezel, D. (2017). Daily activity of the housefly, *Musca domestica*, is influenced by temperature independent of 3' UTR *period* gene splicing. *G3: Genes, Genomes, Genetics*, 7(8), 2637-2649. https://doi.org/10.1534/g3.117.042374

Bell, L. R., Maine, E. M., Schedl, P., & Cline, T. W. (1988). Sex-lethal, a *Drosophila* sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. *Cell*, 5(6), 1037-1046. https://doi.org/10.1016/0092-8674(88)90248-6

Boothroyd, C. E., Wijnen, H., Naef, F., Saez, L., & Young, M. W. (2007). Integration of light and temperature in the regulation of circadian gene expression in *Drosophila*. *PLoS Genetics*, 3(4), e54. https://doi.org/10.1371/journal.pgen.0030054

Brown, J. B., Boley, N., Eisman, R., May, G. E., Stoiber, M. H., Duff, M. O., … Celniker, S. E. (2014). Diversity and dynamics of the *Drosophila* transcriptome. *Nature*, 512, 393-399. https://doi.org/10.1038/nature12962

Collins, B. H., Rosato, E., & Kyriacou, C. P. (2004). Seasonal behavior in *Drosophila melanogaster* requires the photoreceptors, the circadian clock, and phospholipase C. *Proceedings of the National Academy of Sciences of the United States of America*, 101(7), 1945-1950. https://doi.org/10.1073/pnas.0308240100

Dutertre, M., Sanchez, G., Barbier, J., Corcos, L., & Auboeuf, D. (2011). The emerging role of pre-messenger RNA splicing in stress responses: Sending alternative messages and silent messengers. *RNA Biology*, 8(5), 740-747. https://doi.org/10.4161/rna.8.5.16016

Fujita, K., Mondal, A. M., Horikawa, I., Nguyen, G. H., Kumamoto, K., Sohn, J. J., … Harris, C. C. (2009). p53 isoforms Δ133p53 and p53β are endogenous regulators of replicative cellular senescence. *Nature Cell Biology*, 11, 1135-1142. https://doi.org/10.1038/ncb1928

Goto, S. G., & Denlinger, D. L. (2002). Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: *period, timeless, cycle* and *cryptochrome*. *Journal of Insect Physiology*, 48(8), 803-816. https://doi.org/10.1016/S0022-1910(02)00108-7

Heinzen, E. L., Ge, D., Cronin, K. D., Maia, J. M., Shianna, K. V., Gabriel, W. N., … Goldstein, D. B. (2008). Tissue-specific genetic control of splicing: Implications for the study of complex traits. *PLoS Biology*, 6(12), e1000001. https://doi.org/10.1371/journal.pbio.1000001

Jakšić, A. M., & Schlötterer, C. (2016). The interplay of temperature and genotype on patterns of alternative splicing in *Drosophila*

*melanogaster*. *Genetics*, 204(1), 315–325. https://doi.org/10.1534/genetics.116.192310

Jurica, M. S., & Moore, M. J. (2003). Pre-mRNA splicing: Awash in a sea of proteins. *Molecular Cell*, 12(1), 5-14. https://doi.org/10.1016/S1097-2765(03)00270-3

Kannan, S., Halter, G., Renner, T., & Waters, E. R. (2018). Patterns of alternative splicing vary between species during heat stress. *AoB PLANTS*, 10(2), ply013. https://doi.org/10.1093/aobpla/ply013

Kanopka, A., Muhlemann, O., & Akusjarvi, G. (1996). Inhibition by SR proteins splicing of a regulated adenovirus pre-mRNA. *Nature*, 381, 535-538. https://doi.org/10.1038/381535a0

Kucherenko, M. M., & Shcherbata, H. R. (2018). miRNA targeting and alternative splicing in the stress response - Events hosted by membrane-less compartments. *Journal of Cell Science*, 131(4), jcs202002. https://doi.org/10.1242/jcs.202002

Laloum, T., Martín, G., & Duque, P. (2018). Alternative splicing control of abiotic stress responses. *Trends in Plant Science*, 23(2), 140-150. https://doi.org/10.1016/j.tplants.2017.09.019

Liu, H. X., Cartegni, L., Zhang, M. Q., & Krainer, A. R. (2001). A mechanism for exon skipping caused by nonsense or missense mutations in BRCA1 and other genes. *Nature Genetics*, 27, 55-58. https://doi.org/10.1038/83762

Long, J. C., & Caceres, J. F. (2009). The SR protein family of splicing factors: Master regulators of gene expression. *Biochemical Journal*,

417(1), 15-27. https://doi.org/10.1042/BJ20081501

Majercak, J., Sidote, D., Hardin, P. E., & Edery, I. (1999). How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron*, 24(1), 219-230. https://doi.org/10.1016/S0896-6273(00)80834-X

Martin Anduaga, A., Evantal, N., Patop, I. L., Bartok, O., Weiss, R., & Kadener, S. (2019). Thermosensitive alternative splicing senses and mediates temperature adaptation in *Drosophila*. *ELife*, 8, e44642. https://doi.org/10.7554/eLife.44642

Matlin, A. J., Clark, F., & Smith, C. W. J. (2005). Understanding alternative splicing: Towards a cellular code. *Nature Reviews Molecular Cell Biology*, 6, 386-398. https://doi.org/10.1038/nrm1645

Montelli, S., Mazzotta, G., Vanin, S., Caccin, L., Corrà, S., De Pittà, C., … Costa, R. (2015). *period* and *timeless* mRNA splicing profiles under natural conditions in *Drosophila melanogaster*. *Journal of Biological Rhythms*, 30(3), 217–227. https://doi.org/10.1177/0748730415583575

Mount, S. M., & Salz, H. K. (2000). Pre-messenger RNA processing factors in the *Drosophila* genome. *Journal of Cell Biology*, 150(2), F37-F44. https://doi.org/10.1083/jcb.150.2.f37

Nilsen, T. W., & Graveley, B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature*, 463(7280), 457–463. https://doi.org/10.1038/nature08909

Pan, Q., Shai, O., Lee, L. J., Frey, B. J., & Blencowe, B. J. (2008). Deep

surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nature Genetics*, 40, 1413-1415. https://doi.org/10.1038/ng.259

Pozzoli, U., & Sironi, M. (2005). Silencers regulate both constitutive and alternative splicing events in mammals. *Cellular and Molecular Life Sciences*, 62, 1579-1604. https://doi.org/10.1007/s00018-005-5030-6

Shakhmantsir, I., Nayak, S., Grant, G. R., & Sehgal, A. (2018). Spliceosome factors target *timeless* (*tim*) mRNA to control clock protein accumulation and circadian behavior in *Drosophila*. *ELife*, 7, e39821. https://doi.org/10.7554/eLife.39821

Singh, S., Giesecke, A., Damulewicz, M., Fexova, S., Mazzotta, G. M., Stanewsky, R., & Dolezel, D. (2019). New Drosophila Circadian Clock Mutants Affecting Temperature Compensation Induced by Targeted Mutagenesis of Timeless. *Frontiers in Physiology*, 10, 1442. https://doi.org/10.3389/fphys.2019.01442

Tan, S., Wang, W., Tian, C., Niu, D., Zhou, T., Jin, Y., … Liu, Z. (2019). Heat stress induced alternative splicing in catfish as determined by transcriptome analysis. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics*, 29, 166-172. https://doi.org/10.1016/j.cbd.2018.11.008

Zheng, Z. M. (2004). Regulation of alternative RNA splicing by exon definition and exon sequences in viral and mammalian gene expression. *Journal of Biomedical Science*, 11, 278-294. https://doi.org/10.1159/000077096

# Chapter 3

Exploring the unexplored role of *timeless* in temperature compensation and light sensitivity

**Singh S**, Doležel D.

(Unpublished results)

# Summary and Conclusions

This thesis deals with the goal to explore the possible connection of the core clock gene *timeless* (*tim*) in the temperature compensation mechanism in *D. melanogaster*. The goals were accomplished by using several complementary, yet different reverse genetic approaches. In the first chapter of the thesis we describe our set up for a targeted mutagenesis of coding region in *tim* and development of a specific screening protocol targeting temperature compensation mutants. The second chapter utilizes a similar tool, CRISPR/Cas mutagenesis, however, the target was a specific intron, for which temperature-specific retention was postulated, but the exact role was not fully explored. The last chapter describes our systematic approach for TIM research, when a very specific mutant fly was engineered to allow for rescue experiments and for more demanding structural modification of TIM.

These three chapters cumulatively point to several new findings. Our results suggest that short deletions of several amino acids or single amino acid substitutions in the 12$^{th}$ exon lead to strongest temperature compensation defect among other targets in *tim*. It therefore points to the possibility that *tim* contributes to this mechanism by harbouring sites which either itself or by post-translational modifications help to attain a specific conformation required for its interaction with other binding partners. Mutation in those critical sites may thus affect the functionality by either change in the phosphorylation status or the mutation itself disrupts the conformation of TIM required for its interaction with other binding partners (Matsumoto et al., 1999; Wülbeck et al., 2005).

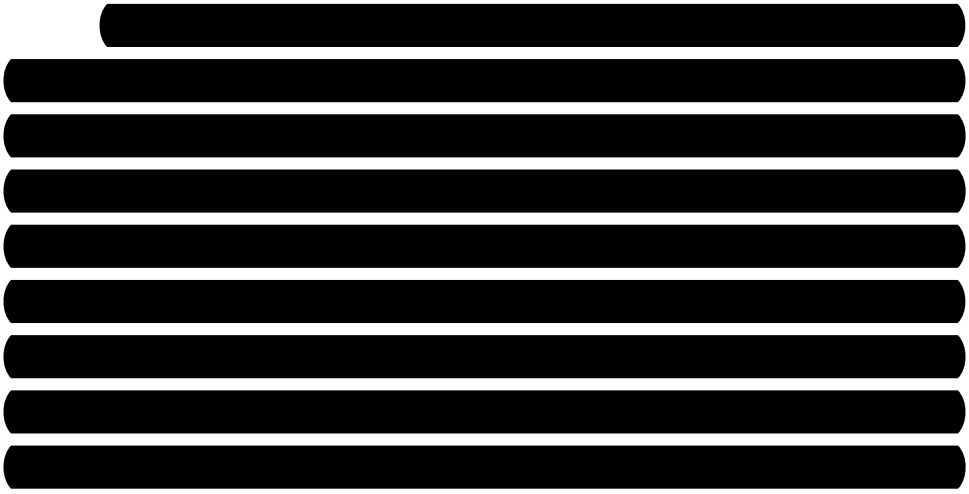
Through different mutant studies the role of post-translational mechanisms such as phosphorylation, ubiquitination in some of the

period determining reactions are known. These processes are known to have a vital role in the timed complex formation, nuclear translocation of the core clock genes and the mutations that disrupt any of these processes may interfere with the temperature compensation. However, when most of these mutant studies unravelled the role of the post-translational mechanisms, some studies reported few post-transcriptional mechanisms also that regulates the levels of the core clock gene mRNAs in *D. melanogaster* in a timely manner. These include thermosensitive alternative splicing in *per*, *tim* mRNA (Boothroyd et al., 2007; Collins et al., 2004; Majercak et al., 1999; Martin Anduaga et al., 2019; Montelli et al., 2015; Shakhmantsir et al., 2018), miRNAs regulated *Clk* mRNA post-transcriptional control (Lerner et al., 2015) and POP2 deadenylase mediated *tim* mRNA stability in *D. melanogaster*.

Therefore, in the second chapter, we addressed the significance of an alternate splicing event resulting into intron retention. The intron retention resulting into a shorter version of *tim*, *tim-tiny*, acts as a mechanism to delay the daily accumulation of TIM in a temperature dependent manner (Shakhmantsir et al., 2018). Although this splicing isoform is quite abundant at 25℃ and 28℃, it is not present as a functional protein (Martin Anduaga et al., 2019; Shakhmantsir et al., 2018). Also, our study of mutants producing truncated *tim-L* due to a premature stop codon in the exon after the retained intron resulted into total arrhythmic behaviour on all recorded temperatures. It thus confirms that *tim-tiny* alone cannot keep the circadian clock running. [redacted]

FRP). This finding corelates to the advance in the clock when the flies rescued with spliced version of *tim* (Shakhmantsir et al., 2018). Therefore, our study point to the possibility that this splicing event is an adaptation for the fine tuning of the clock by regulating the amount of total TIM by *tim-tiny* with respect to high temperatures and suggests that *tim-L* is very essential for the functional circadian clock at least at high temperatures.

These results demonstrating the identification of regions in *tim* important for temperature compensation prompts us to identify the mechanisms associated with the phenotype of the mutants described above. Despite of the amount of time spent to collect these findings, the unavailability of the structure and good antibodies for *tim* makes this study inconclusive. Therefore, we have opted for a rescue strategy where we introduced a docking site in *tim* to manipulate the gene with desired modifications in context of natural *tim* promoter. This system is even suitable to study temperature compensation mechanism which is otherwise temperature dependent in case of UAS/GAL4 system.



[redacted]

Also, TIM consists of two CRY interaction domains and the deleted region includes the majority of the downstream CRY interaction domain, so it is worth to study the role of CRY in temperature compensation mechanism as already described by Wulbeck et al. (2005) in which $TIM^{blind}$ was shown to have reduced degradation by CRY mediated proteosomal degradation.

To conclude, this study established the connection of circadian clock gene *timeless* in the temperature compensation mechanism. The mutants identified in a specific region in *tim* severely compromised the temperature compensation mechanism. The phenotype was consistent from single base substitutions, short deletions to big deletions. Hence, it indicates that *tim* is involved in the temperature compensation through that specific region. However, the exact mechanism is not known but our results suggest that since the region is involved in the interaction with other binding partners such as PER and CRY. Therefore, there might be a possibility that these binding partners are involved in the temperature compensation mechanism.

## References

Boothroyd, C. E., Wijnen, H., Naef, F., Saez, L., & Young, M. W. (2007). Integration of light and temperature in the regulation of circadian gene expression in *Drosophila*. *PLoS Genetics*, 3(4), e54. https://doi.org/10.1371/journal.pgen.0030054

Collins, B. H., Rosato, E., & Kyriacou, C. P. (2004). Seasonal behavior in *Drosophila melanogaster* requires the photoreceptors, the circadian clock, and phospholipase C. *Proceedings of the National Academy of Sciences of the United States of America*, 101(7), 1945-1950. https://doi.org/10.1073/pnas.0308240100

Lerner, I., Bartok, O., Wolfson, V., Menet, J. S., Weissbein, U., Afik, S., … Kadener, S. (2015). *Clk* post-transcriptional control denoises circadian transcription both temporally and spatially. *Nature Communications*, 6(1), 7056. https://doi.org/10.1038/ncomms8056

Majercak, J., Sidote, D., Hardin, P. E., & Edery, I. (1999). How a circadian clock adapts to seasonal decreases in temperature and day length, 24(1), 219-230. *Neuron*. https://doi.org/10.1016/S0896-6273(00)80834-X

Martin Anduaga, A., Evantal, N., Patop, I. L., Bartok, O., Weiss, R., & Kadener, S. (2019). Thermosensitive alternative splicing senses and mediates temperature adaptation in *Drosophila*. *ELife*, 8, e44642. https://doi.org/10.7554/eLife.44642

Matsumoto, A., Tomioka, K., Chiba, Y., & Tanimura, T. (1999). *tim*$^{rit}$ Lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear

localization. *Molecular and Cellular Biology*, 19(6), 4343–4354. https://doi.org/10.1128/MCB.19.6.4343

Montelli, S., Mazzotta, G., Vanin, S., Caccin, L., Corrà, S., De Pittà, C., … Costa, R. (2015). *period* and *timeless* mRNA splicing profiles under natural conditions in *Drosophila melanogaster*. *Journal of Biological Rhythms*, 30(3), 217–227. https://doi.org/10.1177/0748730415583575

Shakhmantsir, I., Nayak, S., Grant, G. R., & Sehgal, A. (2018). Spliceosome factors target *timeless* (*tim*) mRNA to control clock protein accumulation and circadian behavior in *Drosophila*. *ELife*, 7, 1–27. https://doi.org/10.7554/eLife.39821

Wülbeck, C., Szabo, G., Shafer, O. T., Helfrich-Förster, C., & Stanewsky, R. (2005). The novel *Drosophila tim*$^{blind}$ mutation affects behavioral rhythms but not periodic eclosion. *Genetics*, 169(2), 751-766. https://doi.org/10.1534/genetics.104.036244

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