School of Doctoral Studies in Biological Sciences
University of South Bohemia in České Budějovice
Faculty of Science

Functional Analysis of Circadian Clock genes in *Drosophila melanogaster*

PhD Thesis

Nirav Thakkar

Supervisor: David Dolezel, PhD
Institute of Entomology, Biology Centre, CAS
Faculty of Science, University of South Bohemia

České Budějovice 2024

This thesis should be cited as

Thakkar, N., 2024: Functional analysis of circadian clock genes in *Drosophila melanogaster*. PhD Thesis, University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 175 pp.

Annotation

This PhD thesis focuses on the functional analysis of circadian clock genes in the versatile model organism *Drosophila melanogaster*. Application of molecular methods and bioinformatics analysis enabled us to investigate the evolution of clock proteins, create and characterize novel functional mutants of clock genes and explore the temperature compensation property of the clock. Furthermore, this thesis also focused on the advancement of precise genome editing tool in *Drosophila*, by examining CRISPR base editors for germline editing and using them a novel substitution mutant of the core clock gene *timeless* was made. The results presented in this thesis are divided into three parts, (1) Phylogenetic and functional analysis of DOUBLETIME and its vertebrate homologs Casein kinase I delta (CKIδ) and epsilon (CKIε), (2) Investigating germline editing in *Drosophila* using CRISPR-Cas9-based cytosine and adenine base editors (3) Exploring the role of core clock gene *timeless* in temperature compensation of the circadian clock.

Declaration

I hereby declare that I am the author of this PhD dissertation and that I have used only those sources and literature detailed in the list of references.

České Budějovice Nirav Thakkar

08/01/2024

This thesis originated from a partnership between the Faculty of Science, University of South Bohemia, and the Institute of Entomology, Biology Centre of the Czech Academy of Sciences, supporting doctoral studies in the Molecular and Cell Biology and Genetics study program.

Financial support

- Czech Science Foundation (GACR), grant number 22-10088S
- European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no 726049)
- One-year Individual PhD student grant (079/2018/P), Grant Agency of Jihoceska University, 2018-2019, Functional analysis of circadian clock genes in *Drosophila melanogaster*

Acknowledgements

I would like to express my deepest gratitude to my PhD supervisor David Dolezel who offered me a chance to do my doctoral training in his lab. This journey would have not been possible without David's endless patience, trust in my work and frequent suggestions of doing more and more experiments. I remember his first response to my PhD application email where he mentioned that although I am motivated and have research experience, doing a PhD is tough, and requires a lot of hard work and endless patience. Indeed, it was quite a unique experience and all these years have changed my perception about how science works.

This journey would have been even more difficult if there had not been wonderful colleagues in the lab to help me, taking into account even the smallest things in the lab such as how to navigate to the insectarium, and the difficult experiments such as complex cloning. In my initial days, Olina, (my co-supervisor for the short term) trained me in molecular cloning. Samar helped me with the microinjection experiments, finding reagents and talking to him in Hindi made me feel less homesick at times. Vlastik sat beside me, and apart from scientific technical help, I enjoyed long talk sessions with him which ranged from science to environment to world war/politics. Although Bulah's work did not intersect with mine, I enjoyed his jolly nature and insightful scientific discussions at times. I could not miss mentioning Lenka, Marketa, Aska and Magda who were around and happy to help with reagents and fun-filled moments at lab outings. However, the crucial and selfless help came from all the technicians, especially Hanka and Daniela who helped me get enough animals (linden bugs) for experiments, finding the right person to repair the incubators and all the necessary reagents. A part of my time was spent training two wonderful master's students, Adela and Vasek, whose hard work and determination fruited a beautiful publication. Enrico-Ping: You are not forgotten…! Although you guys joined recently, I am

grateful to have you around as you help me not miss too much the people who are not here now and cherish my journey as sometimes you guys remind me of my beginning.

I am also thankful to Dr. Ezio Rosato who hosted me for almost 6 months to get trained in circadian neurogenetics. Also, I could not miss thanking Abhijit Parameswaran and the amazing people in that department who helped me with experiments and my stay there.

Finally, I would like to acknowledge and express immense gratitude to my other half Minal Jain, for not only her moral support but also for taking care of things at home at times when I was working late and finishing writing my thesis recently. I cannot miss thanking my parents who have always believed in me and gave me mental support in difficult times. This list would not be complete without mentioning my best friend Dipak, who has always listened to me in difficult times and given me the most precise and unbiased advice.

List of papers and author's contribution

This thesis is based on the following papers (listed chronologically):

I. **Thakkar, N.**, Giesecke, A., Bazalova, O., Martinek, J., Smykal, V., Stanewsky, R., & Dolezel, D. (2022). Evolution of casein kinase 1 and functional analysis of new doubletime mutants in *Drosophila*. Frontiers in Physiology, 13, 2590. (IF = 4)
DOI: 10.3389/fphys.2022.1062632

Nirav measured and analyzed the locomotor activity data of DBT carboxy-terminal tail mutants. Nirav participated in bioinformatics analysis. Nirav contributed to writing methods/results section and editing/revision of the manuscript. His contribution was 30%

II. **Thakkar, N.**, Hejzlarova, A., Brabec, V., & Dolezel, D. (2023). Germline Editing of *Drosophila* Using CRISPR-Cas9-based Cytosine and Adenine Base Editors. The CRISPR Journal. (IF = 3.7)
DOI: 10.1089/crispr.2023.0026

Nirav and Adela Hejzlarova contributed equally to this work. Nirav performed the molecular cloning of CRISPR base editors and multiple gRNA constructs. Nirav created and molecularly characterized base-edited $tim^{SS308\text{-}9FL}$ mutant. Nirav measured and analyzed the locomotor activity data of the $tim^{SS308\text{-}9FL}$ mutant. Nirav did a part of the white mutagenesis experiment (vasa-Cas9 crosses) and analyzed all white gene editing data. Nirav wrote the manuscript and helped in editing/revision. His contribution was 70%

III. Singh, S., **Thakkar, N.**, Bullo, E., Parameswaran, A., Rosato, E., & Dolezel, D. Unique *Drosophila timeless* mutant with big deletion and severe temperature compensation defect (unpublished results)

Nirav and Samarjeet contributed equally to this work. Nirav performed the molecular cloning of tim_cDNA deletion-like and rescue constructs. Nirav molecularly characterized tim_cDNA fly lines. Nirav measured and analyzed the locomotor activity data of

the respective fly lines in different light-dark conditions. Nirav performed the immunohistochemical analysis of tim_cDNA fly lines at two distinct temperatures, light-dark conditions and time points. Nirav participated in the preparation of the figures and writing methods/results section. His contribution was 90%

Co-author agreement

Dr. David Dolezel, PhD, the supervisor of this PhD thesis and co-author of the listed papers I-III, fully acknowledges the stated contribution of Nirav Thakkar to these manuscripts.

České Budějovice David Dolezel

08/01/2024

Contents

List of abbreviations

aa	amino acid
ABE	adenine base editor
BE	base editor
bdbt	*bride of doubletime* (gene)
BDBT	BRIDE OF DOUBLETIME (protein)
CBE	cytidine base editor
CKIδ	Casein kinase I delta
CKIε	Casein kinase I epsilon
CK2	Casein kinase 2
CKI	Casein kinase I
Clk, CLK	*Clock* (gene), CLOCK (protein)
CRISPR	Clustered regularly interspaced palindromic repeats
cry, CRY	*cryptochrome* (gene), CRYPTOCHROME (protein)
dCRY	*Drosophila* Cryptochrome protein
CT	Circadian time
cyc, CYC	*cycle* (gene), CYCLE (protein)
dbt, DBT	*doubletime* (gene), DOUBLETIME (protein)
DD	constant darkness
DSB	double strand break
eGFP	enhanced green fluorescent protein
EMS	ethyl methane sulfonate
FRP	free-running period
gRNA	guide RNA
h	hour
L:D	Light : Dark
LL	constant Light
l-LNvs	large ventral lateral neurons
LNds	dorsal lateral neurons
mCRY	mammalian Cryptochrome protein
MMEJ	micro-homology mediated end joining
NCBI	National Centre for Biotechnology information
NES	nuclear export signal
NHEJ	non-homologous end joining
NLS	nuclear localization signal
PBS	phosphate buffer saline
per, PER	*period* (gene), PERIOD (protein)
PFA	paraformaldehyde

PP1	protein phosphatase 1
PP2a	protein phosphatase 2a
Q_{10}	temperature coefficient
sgg, SGG	*shaggy* (gene), SHAGGY (protein)
SLIMB	Supernumerary limbs (protein)
s-LNvs	small ventral lateral neurons
SNP	single nucleotide polymorphism
tim, TIM	*timeless* (gene), TIMELESS (protein)
TTFL	transcription-translation feedback loop
ZT	Zeitgeber time
Δ	Deletion

Circadian rhythms

Organisms that evolved under light and dark cycles prevalent in the form of day and night on Earth can anticipate periodic light and temperature changes in their environment. While during evolution, different organisms chose different times of day to be active (viz. diurnal – active during the day, nocturnal – active during the night, crepuscular – active at twilights), it is the genetically determined anticipatory characteristics to time the physiology and behaviour in synchrony with the 24-hour solar day that forms the basis of the circadian rhythms. Perhaps, this is the reason why we are more active until late evenings in long summer days and our bodies literally shut down (signals to sleep) in early evenings in short winter days.

Circadian (derived from Latin where 'circa' – about; 'dies'– day) rhythms are natural oscillations which cycle every 24 hours. Mainly, to characterize any oscillation as circadian it needs to follow three key features of the clock as follows.

1. **Endogenous and free running**: Oscillations should originate within the organism and persist even in constant conditions such as constant darkness, with a period of almost 24 hours. The period of oscillation in these constant conditions is called the free-running period, also otherwise known as tau (τ). This criterion allows us to distinguish circadian oscillations from simple environment responses.
2. **Entrainable:** Oscillations can be reset or changed by exposing them to different external stimuli (light and temperature), a process known as entrainment. A classic example of entrainment which we can relate to is travelling across time zones where initially we feel jetlag but eventually our circadian clock can entrain and be in sync with the local time of travelled region.

3. **Temperature compensated:** Oscillations should maintain their circadian period over a wide range of physiological temperatures. While the rate of biochemical reactions increases with the rise in temperature, circadian oscillations should roughly maintain period of 24 hours despite the change in physiological temperatures known as temperature compensation. This property is very crucial for poikilotherms such as insects. However, it is conserved in homeotherms like mammals too, where the phenomenon can be studied by exposing either isolated tissues or cell cultures to various physiological temperatures.

The first-ever observation of the circadian process was the diurnal movements of tamarind leaves observed by Captain Androsthenes (working under Alexander the Great) in the 4th century BC (Königliche Gesellschaft der Wissenschaften zu Göttingen. n 82086620, n.d.; Theophrastus, n.d.). The very first circadian experiment was conducted by the French scientist Jean-Jacques d'Ortous de Mairan in 1729, where he tried to distinguish the oscillations of the endogenous clock from that of external stimuli by placing *Mimosa pudica* plant in constant darkness. The 24-hour rhythmic patterns in the movement of the *Mimosa* leaves continued even in the constant darkness (DeMairan, 1729). Followed by these in the coming years, circadian rhythms were observed in animals, bees and fruit flies (Patrick and Gilbert, 1896; Forel, 1910; Antle and Silver, 2009). However, it was in 1954 that Colin Pittendrigh experimentally demonstrated that the process of eclosion in *Drosophila pseudoobscura* exhibited circadian behaviour (Pittendrigh, 1954; Bruce and Pittendrigh, 1957). The gateway to the molecular link of circadian behaviour opened in the 1970s when Ron Konopka and Seymour Benzer discovered the first *Drosophila* clock mutant (namely *period* gene; *per*) responsible for behavioural rhythmicity (Konopka and Benzer, 1971). Soon after the discovery of *per* mutant in *Drosophila*, genetics became the prominent tool and wide-scale mutant screens were

employed using different strategies in search of clock mutants in different organisms. Moreover in the 1980s, with the help of emerging recombinant DNA technology, *Drosophila per* gene was mapped independently by the groups of Michael Rosbash and Michael W. Young (Bargiello and Young, 1984; Bargiello et al., 1984; Reddy et al., 1984). Furthermore, around the same time, several clock genes were discovered in prokaryotic and eukaryotic organisms leading to the establishment of models to study circadian oscillation which included *Synechococcus elongatus*, *Neurospora crassa, Arabidopsis thaliana, Drosophila melanogaster and mouse* (Dunlap, 1999; Harmer et al., 2001).

In the 1990s, the molecular details started emerging with the understanding of *per* mRNA and protein cycling leading Paul Hardin, Jeffrey Hall and Michael Rosbash to form the transcription-translation feedback loop hypothesis (Hardin et al., 1990). Subsequently, the *timeless* gene was discovered by Michael Young's group, and over several efforts, they showed that *tim* mRNA also cycles similarly to *per* with the period of 24 hours, TIM protein binds to PER affecting its own nuclear localization and protein levels by inhibiting degradation of PER (Sehgal et al., 1994, 1994; Vosshall et al., 1994; Gekakis et al., 1995; Myers et al., 1995). The era of the late 90s to 2000 saw a remarkable growth in the scientific findings leading to a better understanding of the major molecular mechanism that underlies the circadian clocks in various organisms. Fast-forward to the current date, the fundamental model of the molecular mechanism of the circadian clock has been well established by the leading efforts of Jeffrey C. Hall, Michael Rosbash and Michael W. Young (the awardees of the 2017 Nobel Prize in Physiology or Medicine).

Circadian rhythms have been extensively observed in almost all organisms including animals, plants, fungi and cyanobacteria (Young and Kay, 2001; Bhadra et al., 2017). Unicellular organisms possess stand-alone molecular clocks whereas multicellular organisms (with

differentiated tissues) possess multiple molecular clocks that split the clock function between different tissues and coordinate them to maintain 24-hour rhythms. Mainly in multicellular organisms, the central mechanism of the clock remains includes Input pathways → Central Oscillator → Output Pathways.

Circadian rhythms in *Drosophila*

Circadian rhythms in *Drosophila* are mainly studied using locomotor activity as the output. In 12 hours of light and dark conditions (L:D 12:12), the flies display a bimodal pattern in their locomotor behaviour where they can anticipate light-to-dark as well as dark-to-light transition with their activity peaking at dawn and dusk. Locomotor activity rhythms are based on endogenous clocks and prevail even in constant darkness (DD), although with slightly different patterns. In DD, while the locomotor activity is slightly different (free-runs), the period of the endogenous clock remains almost the same of about 24 hours. Therefore, the precise functionality of the *Drosophila* circadian clock can be easily verified by checking the locomotor activity of the flies. Locomotor activity analysis is usually done by placing a *Drosophila* activity monitor (DAM, Trikinetics, Waltham, USA) loaded with glass tubes (that are prefilled with a single male fly inside with food at one end and cotton plug on another) into the light and temperature-controlled incubator. When the fly moves inside the tube, it cuts the infrared beam which the software counts as a reading. Subsequently, the collective readings are plotted as a locomotor activity graph known as an actogram (Chiu et al., 2010; Pfeiffenberger et al., 2010).

Molecular architecture of Circadian clock

The molecular mechanism of the circadian clock in eukaryotes relies predominantly on the negative transcription-translation feedback

loops (TTFL) (Figure 1). This TTFL involves two distinct elements ; (1) positive and, (2) negative. The positive elements form a heterodimeric complex and are mainly transcription factors that promote the transcription of the negative elements. On the other hand, the heterodimeric complex of negative elements stabilizes and gathers in enough concentrations in the nucleus to inhibit the activity of positive elements, thereby stopping their transcription. Notably, this TTFL model involves a simple transcription-translation process which should be faster than a 24-hour day. The long delay is achieved by multi-step regulation of negative elements which involves their initial destabilization before accumulation and translocation to the nucleus requires dimerization and complex formation. Moreover, both positive and negative elements undergo posttranslational modifications including phosphorylation, dephosphorylation, acetylation, and O-Glc N acylation. The synchrony between all these steps allows for achieving a nearly 24-hour period of one circadian cycle after which the next cycle begins (Bell-Pedersen et al., 2005; Hardin, 2011; Takahashi, 2017).

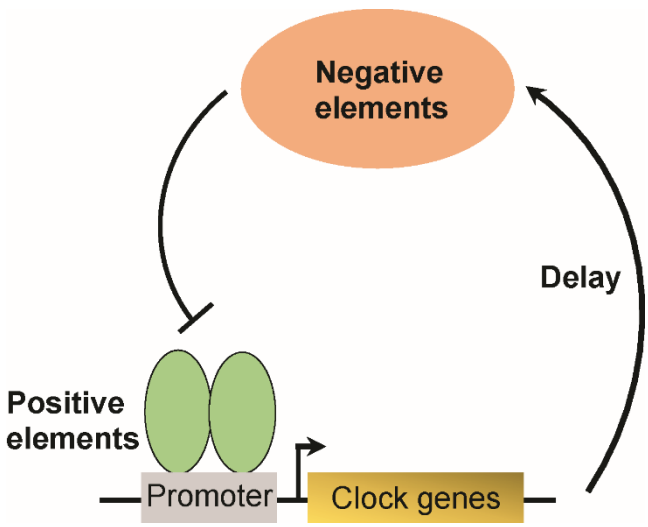

Figure 1: Transcription-translation feedback loop (TTFL) involving delay – A conserved molecular mechanism of circadian clocks (detailed description in the text) adapted from (Takahashi, 2017)

Molecular Mechanism of *Drosophila* Clock

The *Drosophila* molecular clock consists of several interconnected feedback loops that operate via transcription-translation feedback. Amongst them, the core feedback plays a central role. It consists of positive regulators such as *Clock* (*Clk*) and *cycle (cyc)* genes, which as a protein (CLK, CYC) are transcription factors that bind to the promoter region of negative regulators *period* (*per*) and *timeless* (*tim*) genes (Figure 2).

Under 12 hours of light and dark conditions, the transcription of *per* and *tim* happens from around ZT4 to ZT18, when heterodimers of CLK-CYC bind to the E-box motif in the promoter region of *per* and *tim* (ZT refers to Zeitgeber Time, it aids in understanding time in hours during LD 12:12. Here ZT0 means light on time and ZT12 means light off time)(Hao et al., 1997; Allada et al., 1998; Rutila et al., 1998; Darlington et al., 1998). Nearly 6 to 8 hours after the accumulation of *per* and *tim* mRNAs, PER and TIM proteins accumulate in the cytoplasm at about ZT12. This delay in accumulation of PER and TIM is perhaps caused by concerted effects of PER destabilization by DOUBLETIME Kinase (DBT) (homolog of mammalian Casein kinase I epsilon)(Kloss et al., 1998; Price et al., 1998; Kloss et al., 2001) and stabilization by TIM of PER-DBT complexes (Price et al., 1995), thereby resulting into DBT-PER-TIM complexes accumulating in the cytoplasm (Gekakis et al., 1995; Curtin et al., 1996; Zeng et al., 1996). Furthermore, additional phosphorylation of PER by Casein Kinase 2 (CK2) and phosphorylation of TIM by SHAGGY (SGG) assist in the nuclear localization of PER-DBT and TIM (Martinek et al., 2001; Lin et al., 2002; Akten et al., 2003).

To add to the complexity, the phosphorylation of PER and TIM are often removed by phosphatases such as PP2a (protein phosphatase 2a) and PP1 (protein phosphatase 1) which may alter their nuclear localization (Sathyanarayanan et al., 2004; Fang et al., 2007). Entering

the nucleus PER-TIM-DBT complexes promotes CLK phosphorylation by binding to it, thereby removing the CLK-CYC heterodimers from the E-box motifs to inhibit transcription of *per* and *tim* from around ZT18 to ZT4 (Lee et al., 1998, 1999; Bae et al., 2000; Yu et al., 2006; Menet et al., 2010). Lights turn on at ZT0 and promote TIM degradation (Lee et al., 1996; Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). TIM degradation makes PER vulnerable to further phosphorylation by DBT inside the nucleus, ultimately leading to the binding of SLIMB (Supernumerary limbs; E3 ubiquitin ligase), which marks PER for degradation via proteasomal pathway around ZT4 (Naidoo et al., 1999; Kloss et al., 2001; Grima et al., 2002; Ko et al., 2002). With the degradation of PER, gradually hypophosphorylated CLK accumulates and CLK-CYC complexes form, which then bind to E-box motifs of *per* and *tim* to start another cycle of transcription.

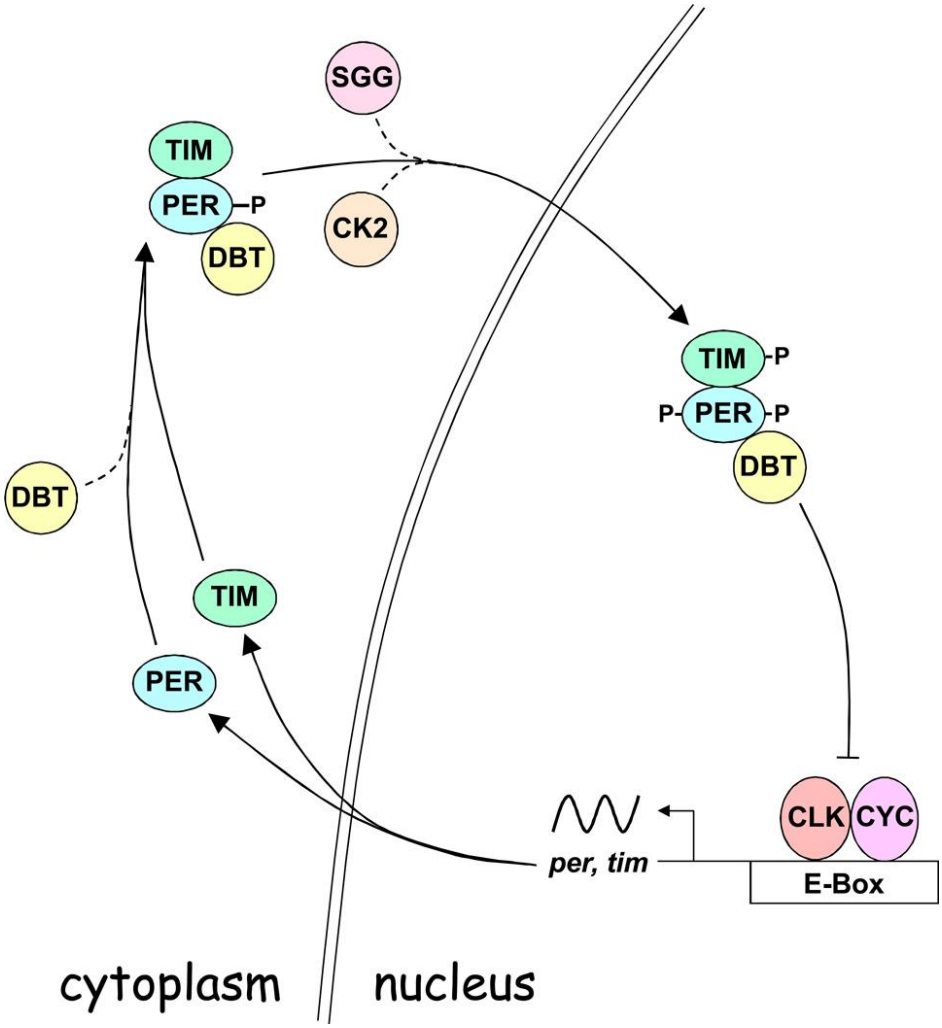

Figure 2: PER-TIM core feedback loop of *Drosophila* circadian clock. (detailed description in the text) adapted from (Hardin, 2011)

Light entrainment of *Drosophila* circadian clock

While the above-described core feedback loop nicely explains the discourse of events that happen during the single oscillatory cycle of 24 hours in LD12:12 conditions, the major light-mediated synchronization in the *Drosophila* clock happens via TIM degradation.

The idea of light-mediated synchronization unfolded in 1996 with the discovery of TIM degradation in the fly heads upon light illumination (Zeng et al., 1996; Myers et al., 1996; Hunter-Ensor et al., 1996). The levels of TIM reduce significantly in the first 30 minutes of the light induction leading to destabilization of the PER. While the light affects the TIM levels uniformly, it is the time when the light pulse is applied that determines the phase of the clock. If a light pulse is given during early evening, it induces a phase delay of the clock which occurs due to replenishment of TIM in a few hours as *tim* mRNA levels are high at that time. However, if the light pulse is given late at night, the phase advancement can be seen in the clock as the low *tim* mRNA levels at that time are not able to replenish the TIM, thereby causing the core-feedback loop to cease earlier than usual. Moreover, if the light pulse is given during the day, it does not have much effect on the phase as TIM levels are normally low during the day.

However, TIM by itself is light insensitive and cannot transduce the photic information. Therefore, the light signal must be detected by the photoreceptor which is then relayed resulting in tyrosine phosphorylation of TIM and its degradation by ubiquitin-proteasomal pathway (Naidoo et al., 1999). The flavoprotein CRYPTOCHROME (CRY) was found to be the exact photoreceptor that detects the light signal (Emery et al., 1998; Stanewsky et al., 1998). Certainly, the interaction of light-activated CRY with TIM ensures the degradation of TIM via the proteasomal pathway (Naidoo et al., 1999; Ceriani et al., 1999; Dissel et al., 2004; Busza et al., 2004). Interestingly, light illumination also results in CRY degradation by the proteasome, but the degradation of CRY happens more slowly than that of TIM. Both TIM and CRY are degraded by JETLAG (JET, F-box protein family) in the presence of light. The preferential degradation by JET occurs due to its higher affinity for TIM than CRY (Peschel et al., 2006, 2009; Koh et al., 2006). Furthermore, light-mediated degradation of TIM also requires the COP9 signalosome

(Knowles et al., 2009). Noticeably, the flies that carry mutations in the COP9 signalosome, *jet* and *cry* were behaviourally rhythmic in constant light conditions (LL)(Emery et al., 2000; Peschel et al., 2006; Dolezelova et al., 2007; Koh et al., 2006; Knowles et al., 2009). Perhaps this indicates that these genes operate in the same pathway to mediate light-dependent synchronization of the circadian clock (Figure 3).

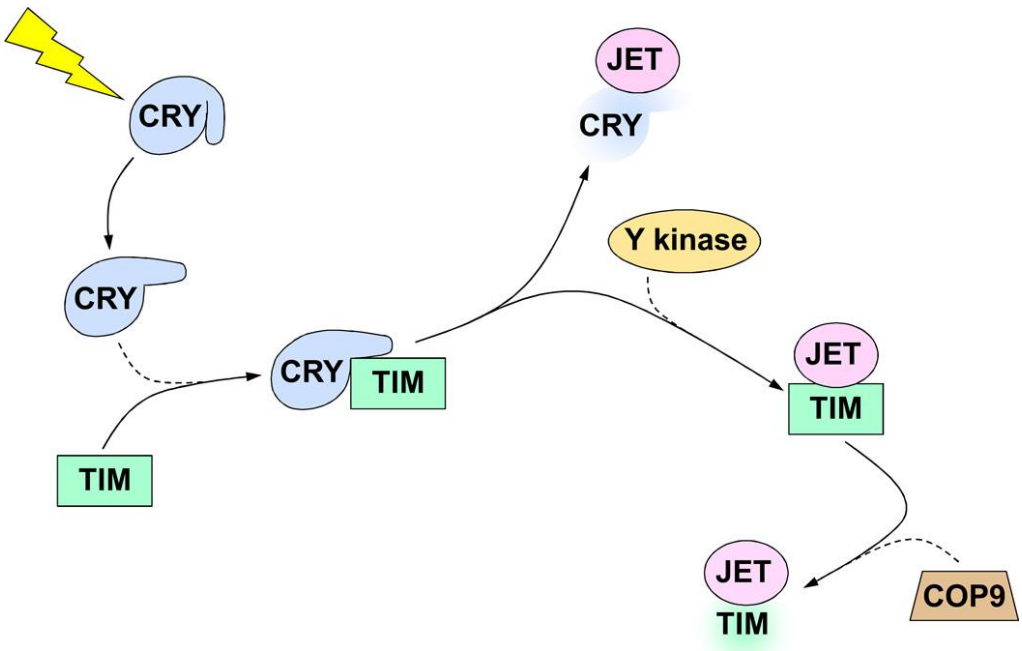

Figure 3: Light entrainment of *Drosophila* circadian clock via CRY-TIM interaction. (detailed description in the text) adapted from (Hardin, 2011)

Temperature compensation in *Drosophila* circadian clock

Besides light, temperature is also a crucial environmental cue that entrains the oscillations of the circadian clock (Buhr et al., 2010; Pittendrigh, 1954). For any chemical or biochemical reaction, the

common convention is that temperature rise will lead to an increase in the rate of reaction thereby reducing the time of reaction (Arrhenius, 1889). On the contrary, despite the clock sensing temperature changes, the speed at which it ticks (i.e. period) almost remains the same known as the temperature compensation property (Pittendrigh, 1954; Zimmerman et al., 1968). This temperature compensation property is highly advantageous for poikilotherms or cold-blooded animals such as insects to maintain their circadian period, regardless of their physiological body temperature altering with changes in environmental temperature. Although temperature compensation is an important feature of circadian clocks, the exact molecular basis behind it is not clearly understood. Albeit, from numerous clock mutations that alter the speed of clock, only a few have been identified that have impaired temperature compensation of the free-running period (Sawyer et al., 1997; Hamblen et al., 1998; Peixoto et al., 1998; Matsumoto et al., 1999; Rothenfluh et al., 2000; Bao et al., 2001; Singh et al., 2019; Giesecke et al., 2023).

Recently, in a targeted mutagenic screen using the CRISPR-Cas9 tool, Singh et al. reported several short in-frame insertion and deletion mutants of *tim* with defects in temperature compensation. Strikingly, these mutations mapped to the C-terminal CRY interaction domain of TIM, a region where *in silico* prediction identified several putative nuclear export signals (NES) (Singh et al., 2019). Similar findings have also been reported for *per* gene where substitution of the single amino acid (PER^{I530A}) altered putative NES leading to longer circadian periods with the rise in temperature (Giesecke et al., 2023). Both these studies point towards the nuclear-cytoplasmic reactions of PER and TIM being temperature-compensated. In addition to this, phosphorylation reactions of PER have also been identified to be crucial in maintaining temperature compensation. Work on mammalian clocks has been seminal towards this understanding and comparative studies in

Drosophila identified PER phosphodegron site that is phosphorylated by DBT leading to PER destabilization and this destabilization is counteracted by phosphorylation of per^{short} (per^{s}) phosphocluster. Notably, stabilization versus destabilization phosphorylation reactions of PER at these sites regulates temperature compensation (Chiu et al., 2011; Zhou et al., 2015; Shinohara et al., 2017; Philpott et al., 2020, 2023; Narasimamurthy and Virshup, 2021).

Apart from this, temperature also affects the splicing pattern of *tim* and *per* 3' untranslated region which remodels the circadian transcriptome and thereby proteome leading to the phase shifts of the circadian oscillations (Majercak et al., 2004; Foley et al., 2019; Martin Anduaga et al., 2019). Additionally, *per* alleles found in natural populations of *Drosophila* also exhibit differential temperature compensation. Mainly, these alleles make PER with variable threonine-glycine repeats (ranging from 14, 17, 20 or 23) that have altered protein conformations at different temperatures thereby affecting its temperature compensation (Costa et al., 1992; Castiglione-Morelli et al., 1995; Sawyer et al., 1997).

Despite several efforts, the clear picture of the involvement of single/multiple molecular processes and the overall mechanism of temperature compensation remains to be elucidated. However, four different models have been suggested based on mathematical prediction and available scientific findings such as (1) Balance hypothesis which states that a balance must exist between period lengthening and shortening reactions resulting from temperature change (Hastings and Sweeney, 1957; Ruoff, 1992; Leloup and Goldbeter, 1997; Ruoff et al., 1997; Kurosawa and Iwasa, 2005; Hong et al., 2007; Bodenstein et al., 2012; François et al., 2012), (2) Critical reaction hypothesis which states that circadian period is regulated by critical reactions which are temperature insensitive (Terauchi et al., 2007; Isojima et al., 2009), (3) Temperature-amplitude coupling hypothesis which states that

temperature sensitive amplitude of circadian oscillations may stabilize the period (Lakin-Thomas et al., 1991; Kurosawa et al., 2017), (4) and lastly, the pathway model which states that there should be dedicated pathway/s for temperature compensation, and the mRNA and protein concentration should rise in a simple manner with the temperature (Kidd et al., 2015).

Network properties of *Drosophila* circadian clock

The molecular mechanism of the circadian clock was initially recognized as a cell-autonomous. However, circadian locomotor behaviour requires the cells to communicate with each other which is possible only via circadian network. Discovery of neuropeptide pigment dispersing factor (*pdf*) mutant established the concept of circadian network (Renn et al., 1999).

The *Drosophila* brain consists of nearly 150 circadian clock neurons. Depending on their neuroanatomical location, they can divided into different groups such as (1) Large and small ventral lateral neurons (l-LNvs, s-LNvs), (2) Dorsal lateral neurons (LNds), (3) three groups of dorsal neurons (DN1s, DN2s, DN3s) (Figure 4). All these groups of neurons express PER-TIM within them and for intercellular communication, they use a variety of neuropeptides and neurotransmitters (Kaneko and Hall, 2000; Helfrich-Förster et al., 2007; King and Sehgal, 2020).

Four s-LNvs and four l-LNvs reside in each hemisphere of the *Drosophila* brain which can be genetically determined by the expression of PDF (Helfrich-Förster, 1995). In addition to this, there also exists a pair of clock cells namely 5th s-LNvs that don't express PDF. The LNvs expressing PDF have a crucial role in the regulation of rest and activity rhythms. When PDF-positive LNvs were either electrically silenced or ablated, flies exhibited arrhythmic behaviour of rest and activity in

constant darkness (DD). In contrast, when *per* expression was restored in LNvs of *per* null mutants, they displayed normal locomotor activity in DD (Renn et al., 1999; Nitabach et al., 2002; Grima et al., 2004; Depetris-Chauvin et al., 2011). Despite PDF-positive LNvs having a central role in rest and activity rhythms, the molecular clocks of individual cell groups in the circadian network need to coordinate to maintain robust rhythmicity. Any mismatch in this coordination leads to complex rhythms (multiple components of rhythmicity with different periods), arrhythmicity, or weak rest and activity rhythms in flies (Yao and Shafer, 2014).

LNds comprise six neurons that reside in each brain hemisphere of *Drosophila*. When neurotransmission is blocked in the LNd group, it results in a higher percentage of arrhythmicity in constant darkness (Guo et al., 2014). Moreover, based on available findings, the circadian network is often classified as a dual oscillator system wherein, PDF positive LNvs regulate the morning peak, while LNds and 5^{th} s-LNvs regulate the evening peak of locomotor activity in fruit flies (Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006; Guo et al., 2014).

DN1s are further subdivided into two anterior (DN1a) and fifteen posterior (DN1p) neurons. In general, DN1s have a role in diverse functions such as entrainment to temperature and light cues and circadian outputs such as mating, sleep and locomotion. Moreover, DN2 also participate in the regulation of temperature entrainment and temperature preference rhythms (the tendency of *Drosophila* to prefer specific temperature at specific time of the day) (Yoshii et al., 2010; Kaneko et al., 2012; Guo et al., 2016; Yadlapalli et al., 2018). In addition to the clock neurons, PER and TIM are also known to be expressed in the glial cells (Zerr et al., 1990). Although it is assumed that the glial cells might have a role in regulating the clock neuron's output, the exact signalling mechanism remains to be elucidated (Ng and Jackson, 2015; Herrero et al., 2017).

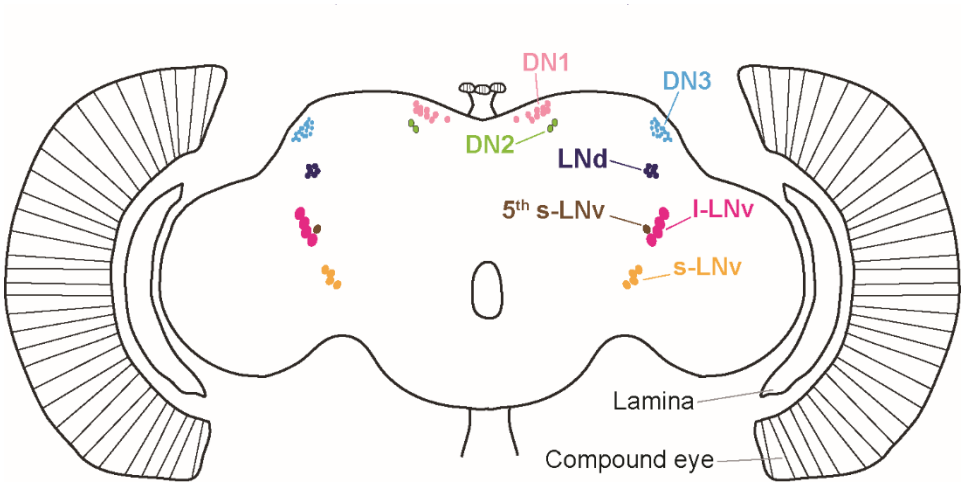

Figure 4: Circadian pacemaker neurons in *Drosophila* brain. (detailed description in the text) adapted from (Rieger et al., 2006)

Genome editing tool: The CRISPR outlook

Forward genetics approaches employing chemical mutagens (ethyl methane sulfonate) and transposon-mediated mutagenesis (*P-elements*) helped in the pioneering work of circadian biology for the potential identification of the genetic basis of the clock (Konopka and Benzer, 1971; Sehgal et al., 1994; Rutila et al., 1996, 1998; Allada et al., 1998; Price et al., 1998; Rothenfluh et al., 2000; Wülbeck et al., 2005). Current approaches incline more towards reverse genetics wherein targeted editing can be done in the genes to further understand their biology (Singh et al., 2019; Joshi et al., 2022).

Since the demonstration of the ability of CRISPR-Cas nucleases to generate a double-stranded break (DSB) at a precise location on DNA in the genome of diverse cells and organisms, it has become the most preferred genome editing tool for reverse genetic approaches. The CRISPR-Cas9 tool employs two components: (1) guide RNA (gRNA) and

(2) CRISPR-associated protein 9 (Cas9 endonuclease). Here, gRNA forms the navigation part which is made up of a scaffold sequence necessary for binding to Cas9 protein and a spacer sequence (~20 nucleotide) which can be replaced with the target DNA sequence. Once inside the nucleus, gRNA and Cas9 form the complex, bind to the target location on the DNA and later the Cas9 endonuclease creates DSB in the DNA. Consequently, the DNA damage is repaired predominantly by non-homologous end-joining (NHEJ) or microhomology-mediated end-joining (MMEJ) pathways. This repair results in several different outcomes such as insertions, deletions, translocation, and frameshift mutations (Figure 5) (Jinek et al., 2012; Cho et al., 2013; Cong et al., 2013; Mali et al., 2013).

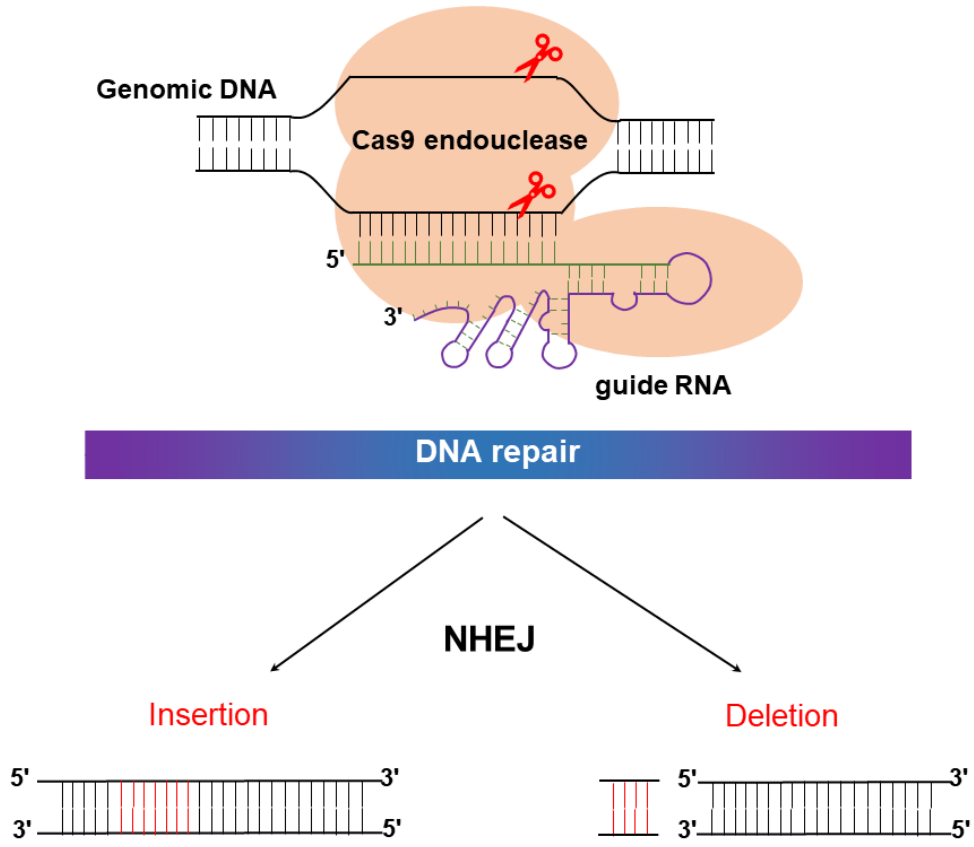

Figure 5: Targeted mutagenesis using CRISPR Cas9 tool. The complex of gRNA and

Cas9 endonuclease binds to the target location on the genomic DNA. Later, Cas9 endonuclease cuts both the strands of DNA. DNA damage is preferentially repaired by error-prone non-homologous end-joining pathways leading to insertion and deletion of nucleotides.

While the CRISPR-Cas9 tool assists in generating targeted mutations, a substantial amount of these mutations are insertions, deletions, and frameshifts. However, to identify the protein motifs and uncover their function, a tool that can precisely manipulate DNA and create single nucleotide modifications is preferable.

In 2016, two groups reported the first development of CRISPR base editors (BE), a tool that permits controllable DNA modifications and the creation of single nucleotide polymorphisms (Komor et al., 2016; Nishida et al., 2016). CRISPR base-editing tool also employs a two-component system which contains a gRNA and modified Cas9 endonuclease. Specifically, modified Cas9 endonuclease encompasses a fusion of DNA deaminase and impairment of its endonuclease activity [either partially (nickase Cas9; nCas9) or totally (dead Cas9; dCas9)]. The base editing begins with gRNA and modified Cas9 forming the complex and binding to the target DNA strand. Next, the respective nucleotide base on the complementary strand is edited by nucleotide deaminase and the change is preserved over the subsequent rounds of the DNA replication. Based on the type of deaminase fused to the Cas9 endonuclease, base editors can be predominantly distinguished into two categories, (1) Cytosine base editors (CBE) which convert C:G DNA base pairs to T:A DNA base pairs. (2) Adenine base editors (ABE) which convert A:T DNA base pairs to G:C DNA base pairs (Figure 6) (Rees and Liu, 2018; Anzalone et al., 2020).

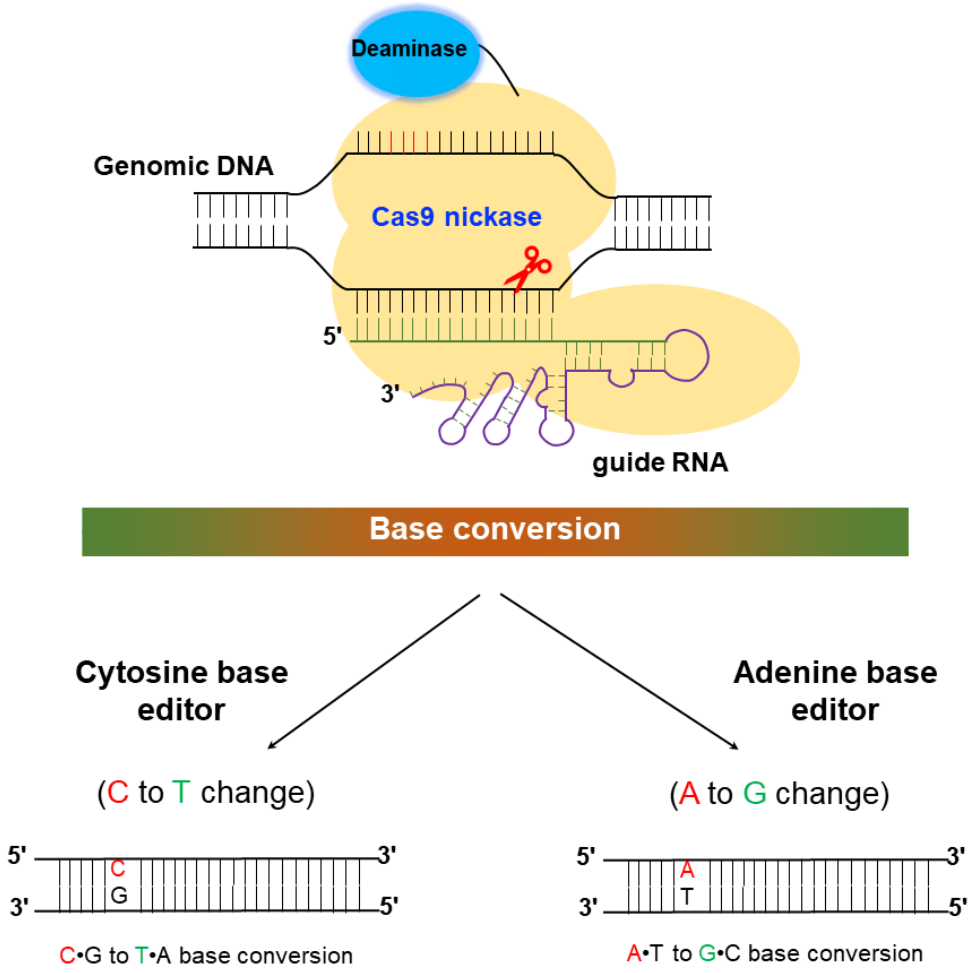

Figure 6: Targeted mutagenesis using CRISPR base editor tool. The complex of gRNA and modified Cas9 endonuclease (detail description in text) binds to the target location on the genomic DNA. Later, modified Cas9 endonuclease (nickase in this case) nicks the single strand of DNA. Subsequently, the nucleotide deaminase edits the nucleotide base on the opposite strand. Nucleotide modification gets fixed when the DNA replication happens. Based on the type of deaminase/base editor fused to the Cas9 endonuclease either cytosine (C) can be edited to thymine (T) or adenine (A) can be edited to guanine (G).

Originally, CRISPR base editors were developed to study human pathogenic point mutations by recreating and correcting such mutations in animal models (Rees and Liu, 2018; Anzalone et al., 2020).

Nonetheless, base editors have been successfully used in several organisms for diverse applications such as targeted random mutagenesis, generating animal models of disease, conferring antibiotic/herbicide resistance, directed protein evolution and modifying non-coding DNA regions in the genome (Kim et al., 2017; Zong et al., 2017; Li et al., 2018; Lu et al., 2018; Carrington et al., 2020; Richter et al., 2020; Song et al., 2020; Doll et al., 2023).

As base editors permit precise single nucleotide editing without DSB, the broad region in the gene can be easily targeted by using multiple gRNAs. This strategy can lead to an array of substitution mutants that can be highly beneficial in facilitating the understanding of potential protein motifs and interaction domains. Perhaps, base editors might serve as an ideal tool for advancing the understanding of protein interaction dynamics in circadian biology.

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Seminal work in the field of circadian biology involved asking fundamental questions such as

- **What are the genetic factors/genes that are responsible for circadian behaviour?**
- **What genes are upstream or downstream in a molecular clock?**
- **What is the overall mechanism of the molecular clock?**

With the significant advancement in the understanding of the *Drosophila* clock in the past decades, the contribution of three Nobel laureates (Jeffrey C. Hall, Michael Roshbash, Michael W. Young) and many others, and comparative studies in *Drosophila* and mouse, the above three questions have been addressed in significant detail.

However, with some questions being answered, there are always new budding questions. Such questions are crucial in pushing forward the field and upgrading our understanding. Below are some of the questions that remain to be addressed.

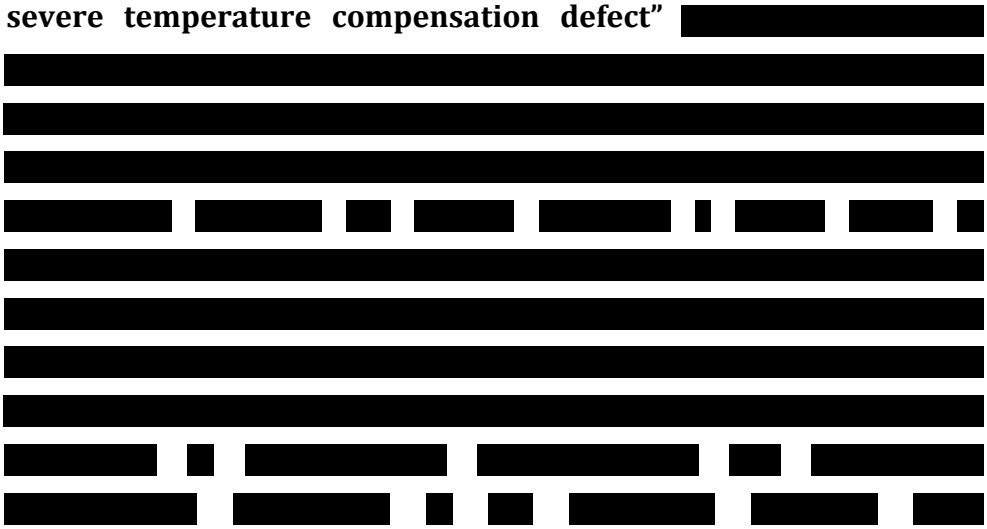
- **What protein motifs are crucial for circadian regulation?**
- **How do post-translational modifications affect the conformation and interaction of circadian proteins?**
- **How do circadian clocks in pacemaker neurons synchronize peripheral clocks?**
- **Do lineage-specific differences in the regulation of circadian oscillations exist, despite the general mechanism of the transcription-translation feedback loop being conserved?**
- **How does the period of the circadian clock achieve temperature compensation, despite the understanding of basic biochemical reactions being temperature sensitive? (the burning question since the establishment of this field).**

This thesis deals with dissecting some of these interesting questions which are described as three original studies in Part1-3.

Part 1 "Evolution of casein kinase 1 and functional analysis of new doubletime mutants in *Drosophila*" explores the evolution of insect DOUBLETIME (DBT) protein and their vertebrate homologs Casein kinase 1 delta (CKIδ) and epsilon (CKIε). In this part, we have dated the origin and divergence of CKIδ from CKIε and identified three independent duplications of CKIδ and CKIε genes. Furthermore, we also identified the conserved regions in DBT that were specific to Diptera and tested some of them by creating functional mutants in *Drosophila*. Interestingly, we found that substituting Lysine 224 in *Drosophila* DBT with acidic residues was homozygous lethal for flies and heterozygous flies had severely impacted the free-running period. Additionally, K224D heterozygous mutants have defect in temperature compensation with slower clocks at higher temperatures. Moreover, we have also identified a unique motif (NKRQK) present only in Diptera which correlated with the presence of FK-506 binding protein, BRIDE OF DOUBELTIME (BDBT) in Diptera (***published in Thakkar et al., 2022***).

Part 2 "Germline Editing of *Drosophila* Using CRISPR-Cas9-based Cytosine and Adenine Base Editors" explores the upgradation of the genome editing tool in *Drosophila*, with the intention of using this tool for targeted mutagenesis of clock genes. In this part, we tested cytosine base editors and adenine base editors for germline editing of the *Drosophila* genome. Interestingly, we found that CRISPR base editing is temperature-dependent in *Drosophila* with maximum editing at 28°C. In addition to this, using the cytosine base editor, we modified the *timeless* gene and scored the substitution mutant with the change of two amino acids ($tim^{SS308\text{-}9FL}$). Moreover, by locomotor activity analysis of the $tim^{SS308\text{-}9FL}$ mutant, we found that the mutant had a disrupted circadian clock with a long free-running period of 29 hours (***published in Thakkar et al., 2023***).

Part 3 "Unique *Drosophila timeless* mutant with big deletion and severe temperature compensation defect" [redacted]



(***unpublished results***).

Part 1

Evolution of casein kinase 1 and functional analysis of new doubletime mutants in *Drosophila*

Thakkar, N., Giesecke, A., Bazalova, O., Martinek, J., Smykal, V., Stanewsky, R., & Dolezel, D.

Frontiers in Physiology, 2022

Circadian clocks of *Drosophila melanogaster* and mammals function via similar mechanism of negative transcription-translational feedback loop (TTFL) using homologous components (Young and Kay, 2001; Stanewsky, 2003). As described earlier, the 24-hour long delay in negative TTFL requires multi-step regulation of negative regulators where the crucial part is protein stability. PERIOD (PER), a member of PAS protein family is a common negative regulator that is present in insects as well as vertebrates (Young and Kay, 2001; Stanewsky, 2003). In *Drosophila*, the stability of PER depends on its interaction with another negative regulator protein TIMELESS (TIM) (Saez and Young, 1996; Meyer et al., 2006). PER-TIM interaction is diminished by *Drosophila* CRYPTOCHROME (dCRY) mediated degradation of TIM in the presence of light (Ceriani et al., 1999; Peschel et al., 2009). In mammals, the stability of PER depends on its interaction with mammalian CRYPTOCHROME (mCRY) (that is present as two paralogs and has light independent circadian function) (Zylka et al., 1998; Kume et al., 1999; Putker et al., 2021). Additional features of negative TTFL involve subcellular localization and shuttling of participating proteins via nuclear localization signal (NLS) and nuclear export signal (NES)(Saez and Young, 1996; Vielhaber et al., 2001; Hara et al., 2011; Jang et al., 2015; Singh et al., 2019; Giesecke et al., 2023). The stability of negative regulators PER and dTIM further depends on post translation modifications which include phosphorylation and dephosphorylation (Mizoguchi et al., 2006; Fang et al., 2007; Akten et al., 2009; Etchegaray et al., 2009; Narasimamurthy et al., 2018).

Among different kinases affecting circadian functions, DOUBLETIME is known to regulate the stability of PER in *Drosophila* (Price et al., 1998; Kloss et al., 2001). In mammals, Casein Kinase1 delta (CKIδ) and epsilon (CKIε) stably interact with PER and play an essential role in circadian function (Lowrey et al., 2000; Xu et al., 2005). DBT and CKIδ/ε are not only sequential homologs, but a substantial amount of

scientific literature has also described their intricate action on PER via phosphoswitch model leading to stability or degradation depending on where the priming phosphorylation happens on PER (Toh et al., 2001; Chiu et al., 2008; Garbe et al., 2013; Zhou et al., 2015; Lam et al., 2018; Narasimamurthy et al., 2018; Top et al., 2018). Despite the shared sequential and functional homology, mammalian CKI homologs could not rescue lethality or circadian function in *Drosophila melanogaster* (Sekine et al., 2008). Such reports assert the lineage-specific differences in circadian clock setup and necessitate systematic analysis involving more diversity apart from two ideal models (*Drosophila* and mouse). Moreover, with the advancement of genomics and transcriptomics in past decade (Misof et al., 2014; Johnson et al., 2018; Kawahara et al., 2019; McKenna et al., 2019; Wipfler et al., 2019), considerable amount of data of diverse species can be easily accessed with a few clicks on NCBI website.

Therefore, in this chapter, we systematically compared the protein sequences of insect DOUBLETIME (DBT) and their vertebrate homologs Casein Kinase1 delta (CKIδ) and epsilon (CKIε). Specifically, our motivation was to understand evolution of these proteins and shed light on the subtle differences which may have a role in differential clock regulation in insects and mammals.

Using systematic phylogenetic analysis, here we dated the origin and the divergence of CKIδ from CKIε and identified nearly three independent events of duplication of the CKIδ/ε in sea lamprey *Petromyzon marinus*, zebrafish *Danio rerio* and the clawed frog *Xenopus laevis*. Furthermore, comparing insect DBT proteins, we identified conserved regions in DBT that were specific to Diptera. Some of these conserved regions were functionally tested in *Drosophila melanogaster*. Especially, replacing Lysine 224 in *Drosophila* DBT with acidic residues conferred quite striking phenotypes. We found that homozygous DBT K224 substitution mutants were not viable, and the free-running period

of the heterozygous mutants was strongly impacted. Furthermore, heterozygous K224D mutants had defect in the temperature compensation of the free running period with slower clock at higher temperatures (a trend that was opposite to that of previously reported mammalian K224D mutants, Shinohara et al., 2017).

Besides this, the *in-silico* analysis revealed interesting feature of the Dipteran DBT sequences which contained NKRQK motif at position 220 to 224. Several recent reports highlight the interaction of *Drosophila* DBT with non-canonical FK-506 binding protein BRIDE OF DOUBLETIME (BDBT)(Fan et al., 2013; Venkatesan et al., 2015; Nolan et al., 2023). Remarkably, the presence of NKRQK motif correlated with the presence of BDBT in Diptera. This enticed the phylogenetic analysis of FK506-binding proteins in insects and representative deuterostomian species which suggested that either BDBT was absent in non-dipteran insects, or it got highly modified. Apart from this, we also identified four new casein kinase 1 genes that were specific to *Drosophila* genus.

frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 14 December 2022
DOI 10.3389/fphys.2022.1062632

OPEN ACCESS

EDITED BY
Ueli Schibler,
Université de Genève, Switzerland

REVIEWED BY
Charalambos P. Kyriacou,
University of Leicester, United Kingdom
Emi Nagoshi,
Université de Genève, Switzerland
Isaac Edery,
Rutgers, The State University of New Jersey–Busch Campus, United States
Charlotte Helfrich-Förster,
Julius Maximilian University of Würzburg, Germany

*CORRESPONDENCE
Ralf Stanewsky,
stanewsky@wwu.de
David Dolezel,
dolezel@entu.cas.cz

SPECIALTY SECTION
This article was submitted to Chronobiology, a section of the journal Frontiers in Physiology

RECEIVED 06 October 2022
ACCEPTED 28 November 2022
PUBLISHED 14 December 2022

CITATION
Thakkar N, Giesecke A, Bazalova O, Martinek J, Smykal V, Stanewsky R and Dolezel D (2022), Evolution of casein kinase 1 and functional analysis of new *doubletime* mutants in *Drosophila*. *Front. Physiol.* 13:1062632. doi: 10.3389/fphys.2022.1062632

Evolution of casein kinase 1 and functional analysis of new *doubletime* mutants in *Drosophila*

Nirav Thakkar[1,2], Astrid Giesecke[3], Olga Bazalova[1], Jan Martinek[1], Vlastimil Smykal[1], Ralf Stanewsky[3]* and David Dolezel[1,2]*

[1]Biology Center of the Academy of Sciences of the Czech Republic, Institute of Entomology, Ceske Budejovice, Czechia, [2]Faculty of Science, University of South Bohemia, Ceske Budejovice, Czechia, [3]Institute of Neuro- and Behavioral Biology, Westfälische Wilhelms University, Münster, Germany

Circadian clocks are timing devices that rhythmically adjust organism's behavior, physiology, and metabolism to the 24-h day-night cycle. Eukaryotic circadian clocks rely on several interlocked transcription-translation feedback loops, where protein stability is the key part of the delay between transcription and the appearance of the mature proteins within the feedback loops. In bilaterian animals, including mammals and insects, the circadian clock depends on a homologous set of proteins. Despite mostly conserved clock components among the fruit fly *Drosophila* and mammals, several lineage-specific differences exist. Here we have systematically explored the evolution and sequence variability of insect DBT proteins and their vertebrate homologs casein kinase 1 delta (CKIδ) and epsilon (CKIε), dated the origin and separation of CKIδ from CKIε, and identified at least three additional independent duplications of the CKIδ/ε gene in *Petromyzon*, *Danio*, and *Xenopus*. We determined conserved regions in DBT specific to Diptera, and functionally tested a subset of those in *D. melanogaster*. Replacement of Lysine K224 with acidic residues strongly impacts the free-running period even in heterozygous flies, whereas homozygous mutants are not viable. K224D mutants have a temperature compensation defect with longer free-running periods at higher temperatures, which is exactly the opposite trend of what was reported for corresponding mammalian mutants. All DBTs of dipteran insects contain the NKRQK motif at positions 220–224. The occurrence of this motif perfectly correlates with the presence of BRIDE OF DOUBLETIME, BDBT, in Diptera. BDBT is a non-canonical FK506-binding protein that physically interacts with *Drosophila* DBT. The phylogeny of FK506-binding proteins suggests that BDBT is either absent or highly modified in non-dipteran insects. In addition to *in silico* analysis of DBT/CKIδ/ε evolution and diversity, we have identified four novel casein kinase 1 genes specific to the *Drosophila* genus.

KEYWORDS

casein kinase 1, doubletime, evolution, circadian clock, temperature compensation, bride of doubletime

Introduction

To cope with and anticipate daily environmental changes, organisms have evolved circadian clocks. These genetically determined time-measuring devices "tick" with a free-running period (τ) close to 24 h (Dunlap, 1999). The circadian clock runs with almost the same τ within the physiological temperature range; this phenomenon, known as *temperature compensation*, seemingly contradicts the basic principles of biochemical reactions (Arrhenius, 1889). At the molecular level, circadian clocks in eukaryotes comprise interlocked negative transcription-translation feedback loops (TTFL; Dunlap, 1999). The positive regulators are transcription factors driving the expression of mRNAs encoding the negative regulators. Once the negative regulators are present in the nucleus, they inhibit their own expression by suppressing the activity of the positive regulator(s). Importantly, the mere transcription-translation process would be much faster than the required 24-h cycle, thus, additional steps delaying the entire process must be involved. Firstly, the negative regulator proteins are initially destabilized, which delays their accumulation. In addition, the translocation of the negative regulators to the cell nucleus might require dimerization with a partner protein, and often larger complexes are formed (Aryal et al., 2017). At a biochemical level, both positive and negative regulators undergo various posttranslational modifications, of which protein phosphorylation is the most prominent. In the end, well-timed depletion of the negative regulators is key for the start of the next cycle and contributes to the resulting τ.

Drosophila and mammalian clock

The circadian clock of mammals and the fruit fly *Drosophila melanogaster* relies on homologous components. The positive regulators CLOCK and BMAL/CYCLE belong to the basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) transcription factors (King et al., 1997; Darlington et al., 1998; Hogenesch et al., 1998; Rutila et al., 1998). PERIOD (PER), which also belongs to the PAS protein family, is a negative regulator shared among vertebrates and insects (Hardin et al., 1990; Zylka et al., 1998).

In *D. melanogaster*, PER interacts with *Drosophila*-type TIMELESS protein (dTIM) (despite the general conservation of the molecular mechanisms and genetic components among various vertebrates and insects, some important differences exist. Furthermore, the gene/PROTEIN names vary in the literature as they were historically evolving. Here, we use the prefix m-for the so-called mammalian type and the prefix d-for the *Drosophila*-type proteins. See the supplementary text for more detailed notes on circadian clock gene terminology).

PER:dTIM dimerization in the cytoplasm is necessary for subsequent nuclear localization of PER and dTIM (Saez and Young, 1996; Meyer et al., 2006). dTIM is an essential component of the fruit fly circadian clock, because *tim* null mutations result in complete arrhythmicity (Sehgal et al., 1994), whereas missense mutations affect τ (Rothenfluh et al., 2000; Wulbeck et al., 2005), and certain *d-tim* mutations affect the temperature compensation of the circadian clock (Matsumoto et al., 1999; Singh et al., 2019). Furthermore, dTIM is a key component of the light-mediated synchronization in *Drosophila* (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996), which involves light-dependent interaction with *Drosophila*-type CRYPTOCHROME (dCRY) (Ceriani et al., 1999; Peschel et al., 2009). dCRY serves as a deep brain circadian photoreceptor (Emery et al., 2000) with no impact on the behavioral rhythmicity in constant-dark conditions (DD) at ambient temperature (Stanewsky et al., 1998), although *d-cry* depletion reduced rhythmicity at 18°C (Dolezelova et al., 2007). Interestingly, *d-cry* mutations abolish transcriptional oscillations in peripheral clocks, which allowed the identification of this mutant in a luciferase reporter-based screen (Stanewsky et al., 1998). In mice, mammalian-type CRYPTOCHROME (mCRY) is present as two paralogous and closely related proteins, that are essential for (light-independent) clock function, while dimerizing with one of the three mammalian PER proteins (Zylka et al., 1998; Kume et al., 1999; Putker et al., 2021).

An important feature of the negative TTFL is the temporal regulation of subcellular localization of participating proteins. In addition to nuclear localization signals (NLS), some circadian clock proteins also contain nuclear export signals (NES) (Saez and Young, 1996; Vielhaber et al., 2001; Ashmore et al., 2003; Yildiz et al., 2005; Hara et al., 2011; Saez et al., 2011; Jang et al., 2015; Singh et al., 2019; Giesecke et al., 2021). Thus, the resulting nuclear import/export strongly affects the suppression potential of the negative feedback loop and thereby τ. The stability and subcellular localization of the negative complex, such as PER and dTIM, is regulated by posttranslational modifications (Li et al., 2019; Crosby and Partch, 2020), including phosphorylation and dephosphorylation by several kinases and phosphatases (Sathyanarayanan et al., 2004; Leloup 2009; Reischl and Kramer, 2011; Agrawal and Hardin, 2016; Narasimamurthy and Virshup., 2021). One of the most explored circadian clock kinases is DBT which was first identified as a clock component in a Drosophila screen when the short- (DBT^S) and long- (DBT^L) free-running period mutants were identified (Price et al., 1998; Kloss et al., 1998, Figure 8). However, as it turned out later, *dbt* is also known as *discs overgrown*, a gene which had been discovered for its role during development (Jursnich et al., 1990; Zilian et al., 1999). Mammalian homologs of DBT are CKIδ/ε, which were shown to be essential for the clock in the hamster, human, and mice (Lowrey et al., 2000; Xu et al., 2005; Meng et al., 2008). The interaction between this kinase and PER is remarkably stable (Kloss et al., 2001; Lee et al., 2001; Aryal et al., 2017). Overexpression of either DBT^L or DBT^S variants in *Drosophila* resulted in the same τ as was produced by the corresponding alleles of the endogenous gene (Muskus et al., 2007), whereas *in vitro* studies using non-physiological substrates

implied, surprisingly, that both mutants have reduced kinase activity (Kivimaë et al., 2008; Venkatesan et al., 2019). Furthermore, unlike most enzymes, CKIδ/ε activity is temperature insensitive (Isojima et al., 2009), but paradoxically, the hamster CK1ε^{tau} mutant is a temperature compensation mutant (Tosini and Menaker, 1998). The conundrum started to unravel in the context of the PER phosphorylation pattern elicited upon the action of multi-kinase hierarchical activities identified in several model organisms (Xu et al., 2007; Ko et al., 2010; Chiu et al., 2011; Lam et al., 2018). The current phosphoswitch model involves two competing phosphorylation sites on mouse (*Mus musculus*) PER2, the phosphodegron and the FASP (familial advanced sleep phase, Toh et al., 2001) sites, which regulate PER2 stability in opposing ways (Zhou et al., 2015; Masuda et al., 2020). Thus, the temperature-sensitive phosphoswitch slows down PER2 degradation at higher temperatures, resulting in a global temperature-compensated system. Somewhat similar, multiple phospho-clusters are detected on *Drosophila* PER, which cumulatively contribute to PER stability and transcriptional repressor activity (Chiu et al., 2008; Kivimaë et al., 2008; Garbe et al., 2013; Top et al., 2018). Therefore, the phosphoswitch mechanism might be conserved across species, even though the details differ, as a phosphodegron with functionally heterogeneous sites was recently reported for *Drosophila* (Joshi et al., 2022).

Both in mammals and *Drosophila*, the PER phosphorylation pattern is defined by the synergistic action of multiple kinases (see the text above), phosphatases (Sathyanarayanan et al., 2004; Fang et al., 2007; Reischl and Kramer, 2011), and some additional post-translational modifications, such as O-GlcNAcylation and acetylation (Kaasik et al., 2013; Li et al., 2019). The PER phosphorylation dynamics is regulated by yet another level of complexity, as is indicated by the distinct capacity of CKIδ splice isoforms. CKIδ1 and CKIε (both similar in the last 16 amino acids of their carboxy-terminal tails, here abbreviated as "C-terminal tails") are more active in priming kinase activity at the FASP site, whereas CKIδ2 is more potent in priming the degron site (Fustin et al., 2018; Narasimamurthy et al., 2018). The CKIδ/ε C-terminal tail autophosphorylation inhibits its kinase activity (Graves and Roach, 1995; for review see Narasimamurthy and Virshup, 2021). As was shown for *Drosophila* DBT, the C-terminal tail stabilizes interactions between the kinase and the substrate, while the C-terminal tail autophosphorylation inhibits substrate binding (Dahlberg et al., 2009; Fan et al., 2015). Furthermore, two residues on the DBT kinase domain influence its affinity to PER (Dahlberg et al., 2009). However, no splicing isoforms of DBT exist in *Drosophila* as *dbt* is an intronless gene in this species.

The temperature-independent activity of CK1δ/ε was connected to sequence motifs close to the active site of the kinase, where Lysine 224 was identified as key for the temperature-compensated primed phosphorylation (Shinohara et al., 2017). Importantly, the K224D mutation in CK1δ shortens τ and affects temperature compensation in the mammalian system *in vitro*. Notably, the corresponding region of *Drosophila* DBT was systematically explored by Venkatesan et al. (2019) who identified a second NLS in positions 220–224. This region in DBT is further important for its interaction with BRIDE of DOUBLETIME (BDBT) (Venkatesan et al., 2015), a non-canonical FK506-binding protein with tetratricopeptide repeats that might promote the assembly of larger protein complexes (Fan et al., 2013).

Although the circadian clock is in general conserved among bilaterian species, some notable variations in the PER/dTIM/dCRY/mCRY feedback exist with some functional implications (Kotwica-Rolinska et al., 2022a). Therefore, we decided to explore and define the variability in insect DBT proteins. As a reference, we analyzed deuterostomian homologs of DBT and dated the origin and separation of CKIδ from CKIε. Furthermore, we have identified four novel casein kinase I genes specific to the *Drosophila* genus. We identified conserved regions in DBT specific to Diptera, functionally tested some of them in *D. melanogaster*, and analyzed their impact on temperature compensation of the circadian clock.

Materials and methods

Recent progress in genome and transcriptome sequencing (Misof et al., 2014; Johnson et al., 2018; Kawahara et al., 2019; McKenna et al., 2019; Wipfler et al., 2019) allowed us to systematically explore casein kinases and FK506-binding proteins across all major insect orders. In essence, we applied an approach similar to that of Smykal et al. (2020), when multiple rounds of Basic Local Alignment Search Tool (BLAST) searches followed by fast phylogenetic analyses were conducted to retrieve evolutionary informative sequences from the genomes and transcriptomes of all major insect lineages. Although a reasonable collection of sequences could be retrieved from the protein database using BLASTP algorithm, more detailed and taxon-focused TBLASTN searches (search in translated nucleotide databases using a protein query) were used to explore transcriptome shotgun assemblies (TSAs). Multiple query sequences were tested in all searches described above (fruit fly *Drosophila melanogaster* DBT, firebrat *Thermobia domestica* DBT, and house mouse *Mus musculus* CKIε/CKIδ). For well-annotated genomes (zebrafish *Danio rerio*, African clawed frog *Xenopus laevis*, *M. musculus*, human *Homo sapiens*, etc.), all protein variants were retrieved directly from gene models. To retrieve non-DBT/CKIε/CKIδ kinases, multiple rounds of BLASTP and TBLASTN were performed. To test whether *Drosophila*-specific CKI genes (CG9962, CG2577, CKIalpha-like I, and CKIalpha-like II) could be identified outside of *Drosophila*, TBLASTN was performed in TSA of all insects with the exclusion of the *Drosophila* genus (NCBI:

txid7215). In addition, reciprocal BLAST searches were performed when the identified sequence served as a query in the next rounds of BLASTs. Additional *dbt* sequences were obtained by PCR and 3′RACE from the housefly *Musca domestica* (Bazalova and Dolezel, 2017) and *Chymomyza costata* (Kobelkova et al., 2010), with support from Illumina-based transcriptome (Poupardin et al., 2015). See Supplementary Tables S1, S2 for accession numbers.

To reconstruct the evolution of BDBT, all FK506-binding protein homologs were retrieved from *D. melanogaster*, the monarch butterfly *Danaus plexippus*, the red flour beetle *Tribolium castaneum*, the brown marmorated stink bug *Halyomorpha halys*, and *M. musculus*. Then, multiple rounds of order- and species-specific searches in insects were employed and fast phylogenetic analyses performed. First, proteins were aligned using the algorithm MAFFT E-INS-i in Geneious 11 (Biomatters). Then, a FAST tree algorithm in Geneious 11 (Biomatters) was used to infer preliminary trees and identify duplicates. For detailed analyses, protein sequences were aligned using MAFFT algorithm with the E-INS-i multiple alignment method and the BLOSUM80 scoring matrix, and the trees were inferred using RAxML maximum likelihood GAMMA-based model and the bootstrap values calculated from 100 replicates (both as a package of Geneious 11 software, Biomatters). The datasets consisted of 239 sequences used for CKI evolution in Figure 2, whereas 31 sequences were used for vertebrate-specific duplication analyses (Figure 4), and 280 sequences were used for BDBT/FK506-binding proteins (Figure 5).

Pyrrhocoris apterus Oxford nanopore technology mRNA sequencing

Details of Oxford Nanopore Technology (ONT) transcriptome sequencing will be described elsewhere. Briefly, *P. apterus* brains and other tissues were dissected and poly A+ mRNA was isolated using Dynabeads mRNA DIRECT Kit (Life Technologies) according to the manufacturer's instructions and 100 ng of the polyA+ mRNA was then reverse-transcribed, turned to double-stranded DNA, and the sequencing adaptors were added using PCR-free Direct cDNA Sequencing kit (SQK-DCS109; Oxford Nanopore Technology) according to the manufacturer's instructions. The library was immediately sequenced on a MinION device (Oxford Nanopore Technology). Base calling was performed after the run using Guppy 3.6.0 at a high-accuracy setting. Obtained tissue-specific transcriptomes were used in exhaustive searches using *P. apterus dbt* mRNA sequence as a query. All *dbt* transcripts were retrieved, manually inspected, and mapped to the in-house *P. apterus* genome (hybrid assembly of Illumina and ONT data, which will also be published separately), and a *dbt* gene model was built. All *dbt* transcripts were sequentially mapped to four defined individual *dbt* isoforms and only reads unequivocally distinguishing specific *dbt* isoform (protein)-coding sequences were counted.

Phosphorylation prediction

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) mutants

The target site was designed to induce a double-strand strand brake in the C-terminal tail coding part of *dbt* gene. Two gRNA sequences (PAM, which is not part of the gRNA, is shown in square brackets) targeting GCGATGCTGGGC GGCAATGG[AGG] and GTCGGCCTTCGATACGGATG [CGG], respectively, were prepared from custom-synthesized oligonucleotides and cloned into pBFv-U6.2 (Kondo and Ueda, 2013) obtained from fly stocks of National Institute of Genetics, Japan (NIG-FLY). Plasmids were injected into y^1 v^1 *P{nos-phiC31\int.NLS}X; attP40 (II)* (NIG-FLY#: TBX-0002) flies with docking site on the second chromosome, transformants identified by eye color rescue, and balanced by y^2 cho^2 $v^1/Y^{hs\text{-}hid}$; *Sp/CyO* (NIG-FLY#: TBX-0008).

Flies expressing Cas9 specifically in germ cells (nos-Cas9) from the second chromosome insertion (NIG-FLY#: CAS-0001; y^2 cho^2 v^1; *attP40{nos-Cas9}/CyO*) were crossed with U6gRNA-encoded transgenic strains (also located on the second chromosome). Resulting F1 offspring thus expressed both gRNA and CAS9 on second chromosomes, which potentially targeted the *dbt* gene located on the third chromosome and induce insertions and deletions as a result of the non-homologous-end-joining (NHEJ) mechanism. The resulting F1 offspring were crossed to y^2 cho^2 v^1; *Pr Dr/TM6C, Sb Tb* (NIG-FLY#: TBX-0010) to balance the modified third chromosomes with TM6C. Individual F1 flies were used in heteroduplex mobility shift assay (Kotwica-Rolinska et al., 2019) to identify flies with the highest degree of mosaicism in the targeted *dbt* locus, thus, the crosses with the highest frequency of NHEJ-induced mutants were identified. From these selected crosses, F2 males and females with third chromosome balancer were individually crossed back to y^2 cho^2 v^1; *Pr Dr/TM6C, Sb Tb* flies (NIG-FLY#: TBX-0010) to establish lines with identically modified third chromosomes. Mutated region was identified by polymerase chain reaction (PCR) and sequencing.

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 *dbt* K244 site. To avoid off-target cleavage optimal target sites were identified using CRISPR target finder (http://flycrispr.molbio.wisc.edu/tools). One gRNA target was chosen that was 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 with 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.

Donor plasmids that contain the desired *dbt* mutations and all elements necessary for homologous recombination were constructed in 3 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 from nos-Cas9 flies (Port et al., 2014) using outside primers dbtBMHRF and dbtBMHRR in combination with respective internal primers. Outside primers dbtBMHRF and dbtBMHRR contain a 15 bp overhang for In-Fusion cloning that is homologous 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 a silent SalI site was introduced that can be used to screen for transformants. The two fragments (5′ homology arm and 3′ homology arm) were 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 either the K224D or the K224E mutation, respectively. 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 PAM site mutations to avoid unwanted Cas9 cleavage within the donor plasmid. See Supplementary table S3 for a detailed list of all primers.

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 were 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*; +; *Dr/TM3* flies to balance 3rd chromosome modifications. Individual male and female flies from this cross were crossed again to *y w*; +; *Dr/TM3*. After letting the females lay eggs for 3–5 days, adult transformant flies were used for molecular screening.

Molecular screening in HDR experiments

In general, a total of 95 flies for each mutation were screened using PCR in combination with restriction digests. A ~800 bp target locus was amplified by PCR using genomic DNA from individual flies. 20 units of SalI were then added to half of the PCR reaction and incubated for 2 h at 37°C. The resulting products were analyzed on agarose gels. The remaining PCR product of samples that showed digested products of the correct size were then used for sequencing to verify the presence of the desired mutations.

Locomotor activity recordings and analysis

No homozygous flies could be obtained for both K224 *dbt* mutations and both stocks are balanced over TM3. Thus, for behavior experiments, flies harboring the *dbt* mutations were crossed against *y w* controls and only flies without TM3 were tested. For *dbt* C-terminal tail mutants, homozygous flies were tested.

Two to four-day old males were loaded into glass tubes containing 5% sucrose in 2% agar and loaded into the DAM2 TriKinetics system (Waltham, MA) and locomotor activity was recorded as previously described (Pfeiffenberger et al., 2010). K224 mutant flies were exposed to 12 h Light: 12 h Dark regime (LD) for 3 days, followed by 5–7 days in constant darkness (DD) to assess their free-running periods at constant temperatures of 18°C, 25°C, or 29°C. Period length and their significance (RS values) were determined using autocorrelation and Chi-square periodogram analysis functions of the fly toolbox implemented in MATLAB (MathWorks) (Levine et al., 2002). Period values with associated RS values ≥1.5 were considered rhythmic (Levine et al., 2002).

Two to four-day old C-terminal tail mutant males were loaded into the DAM2 TriKinetics system as described above, exposed to LD for 5 days, followed by 10 days in DD to assess their free-running periods at constant temperatures of 17°C, 20°C, 25°C, or 28°C. To determine τ during the first 10 days in DD, Lombe-Scargle periodogram analysis was performed using ActogramJ (Schmid et al., 2011) and double-plotted actograms were eye inspected in parallel.

Results

Drosophila DBT diverges both from mammalian and from ancestral insect homologs

Although mammalian CKIε, CKIδ, and *Drosophila* DBT are conserved components of the circadian clock, the mouse *CKIε*

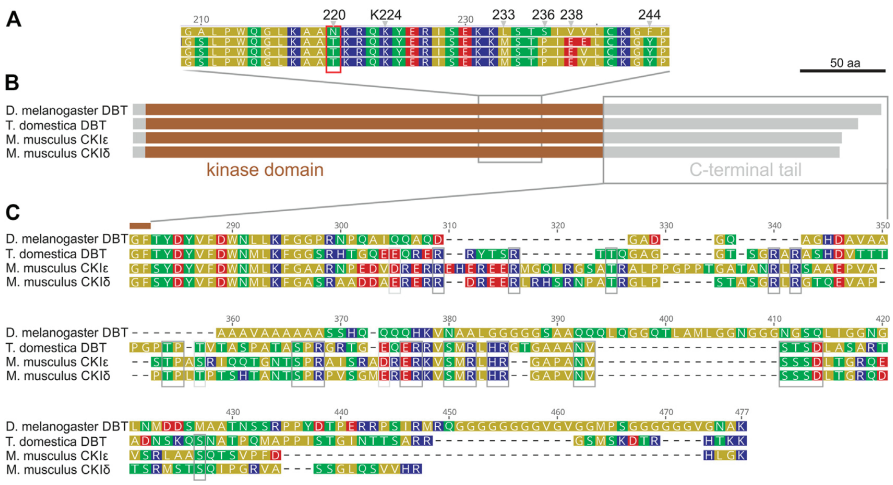


FIGURE 1
Drosophila melanogaster DBT differs from DBT of ancestral insect *Thermobia domestica* and both mouse (*M. musculus*) homologs, CKIε and CKIδ. **(A)** A detail of the kinase domain with highlighted conserved Lysine K224 and *Drosophila*-specific differences: N220, L233, S236, V238, and F244. **(B)** A schematic depiction of proteins with highlighted kinase domain, N- and C-terminal tails, and positions of detailed alignments shown in panels **(A,C)**. **(C)** Detail of the C-terminal tail, where grey boxes indicate residues conserved among CKIε, CKIδ, and *T. domestica* DBT.

sequence did not rescue either the lethality or the rhythmicity of *dbt*-deficient *Drosophila* (Sekine et al., 2008). Therefore, to identify the key differences, we compared the protein sequences of mouse CKIε and CKIδ, *Drosophila melanogaster* DBT, and DBT of the most basal insect, the firebrat *Thermobia domestica* (Figure 1). All four proteins consist of the conserved casein kinase domain with a substantial C-terminal tail, whereas the N-terminal extension is minimal. However, a detailed inspection revealed two major differences. Within the kinase domain, the region 210–244 of *D. melanogaster* DBT differs from all three sequences. Notably, *D. melanogaster* DBT contains Asparagine (N) instead of Threonine (T) in position 220, which contrasts with DBT homologs from *Thermobia*, mouse, (Figure 1A), and more distant kinases. A recent study indicates that autophosphorylation of T220 influences substrate specificity (Cullati et al., 2022), thus the N at 220 prevents this posttranslational regulatory modification. Remarkable sequence divergence is observed in the C-terminal tail. Although the tail contains positions conserved even among both mouse CKI sequences and *T. domestica* DBT, surprisingly, the tail sequence is quite different in *Drosophila* with major insertions and deletions compared to the mammalian and firebrat sequences. These data indicate that during insect evolution DBT acquired substantial changes present in recent *D. melanogaster* DBT.

Evolution of casein kinases in insects and deuterostomia

To be able to perform a comprehensive analysis of DBT/CKIε/CKIδ evolution, we first explored the phylogeny of casein kinases I in insects and used representative deuterostomian species as a reference. Tau-tubulin kinase served as an outgroup. CKI formed five distinct clusters (Figure 2A and Supplementary Figure S1): DBT/CKIε/CKIδ, CKIα, CKIγ, and two additional clusters not assigned to a specific CKI-type. These two clusters, provisionally labeled as CG9962 and CG2577, seem to be specific to the *Drosophila* genus, as no representative was found even in the dipteran genera *Musca* or *Ceratitis*. Similarly, two *Drosophila*-specific clusters are branching at the base of CKIα; thus, we use the provisional terms CKIα-like I and CKIα-like II (see Supplementary Table S1 for all non-DBT acc. numbers and Supplementary Table S2 for DBT, CKIε, and CKIδ acc. numbers). In line with the observed phylogenetic clustering of CKI, differences were identified in the N- and C-terminal tail lengths (Figure 2B) and in the sequence motifs within the kinase domain, including the activation loop (Philpott et al., 2020) and residues N275 and R279, which are responsible for enhanced substrate-specific binding to DBT (Dahlberg et al., 2009) (Figure 2C).

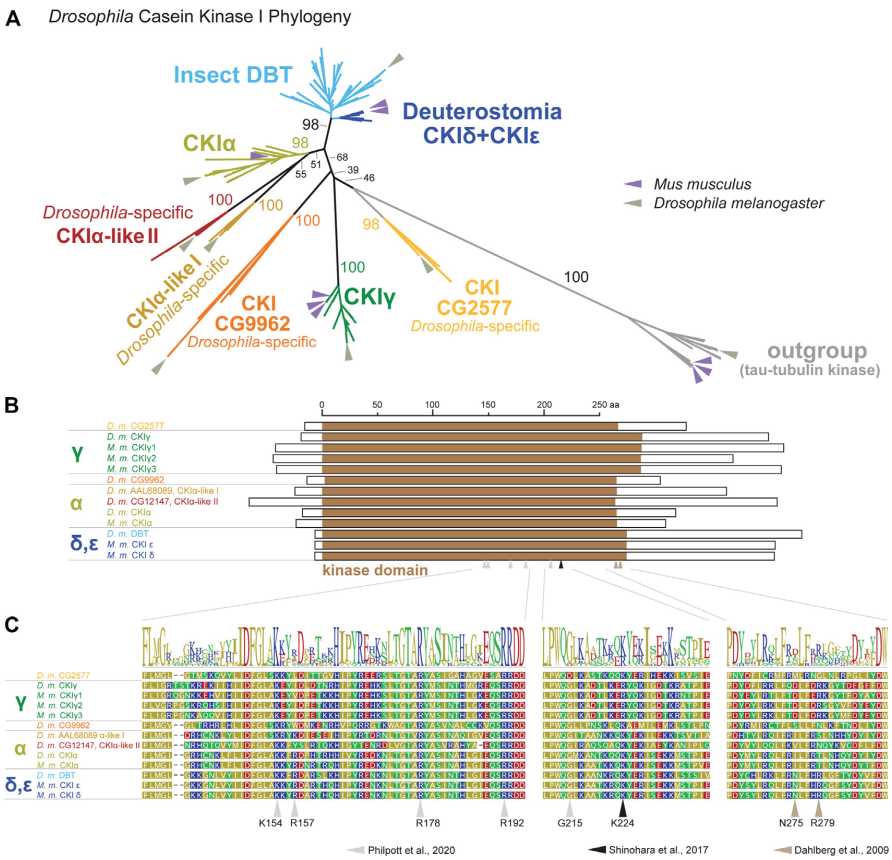


FIGURE 2
Phylogeny of bilaterian Casein kinase I (CKI) reveals seven clearly separated CKI-coding genes in fruit flies (*Drosophila* genus). **(A)** A tree illustrating relatedness among CKI proteins (values above branches indicate bootstrap support), in which well-separated clusters are color-coded. Insect DBTs (cerulean) branch together with deuterostomian sequences (cobalt blue) including CKIε and CKIδ from fish, amphibia, reptiles, and mammals. Besides well-established isoforms CKIα and CKIγ (the latter encoded by *gilgamesh* in *Drosophila*), four *Drosophila* genus-specific clusters were detected. Two of them branch at the base of CKIα and are therefore labeled as CKIα-like I (peanut) and CKIα-like II (cinnamon brown). Two additional clusters are separated from established CKI isoforms and are labeled according to the *D. melanogaster* nomenclature as CG9962 (orange) and CG2577 (apricot). Tau-tubulin kinases (a.k.a. *asator* in *D. melanogaster*) serve as an outgroup. Positions of the fruit fly *D. melanogaster* and the mouse *Mus musculus* proteins are highlighted by arrows in sage green and lavender, respectively. In established CKI terminology, the term isoform (α, γ, δ, ε) is used to refer to kinases encoded by distinct genes, although some of these genes might also encode different splice variants (for clarity, we use the term "splicing isoforms"). Multiple arrows in the mouse refer to gene multiplications, not to splicing isoforms. The phylogenetic analysis strongly supports the existence of the new groups of CKI genes in *Drosophila* and confirms already established groups. However, the relationship among CKI groups is sometimes poorly supported and the tree should not be interpreted as a focused analysis of CKI history. The tree was inferred using RAxML maximum likelihood of 239 protein sequences (final GAMMA-based score of the best tree -72993.763796) using Geneious 11 software (Biometters). Bootstrap support was calculated from 100 replications. See Supplementary Tables S1, S2 for accession numbers of analyzed sequences. **(B)** Schematic illustration of CKI proteins with highlighted kinase domain (brown), N- and C-terminal tails are shown as empty rectangles. **(C)** Details of protein alignment with highlighted residues that are important for the function of CKIδ (Shinohara et al., 2017; Philpott et al., 2020) and DBT (Dahlberg et al., 2009).

Dbt, CKIε, and CKIδ genes in insects and deuterostomia

Having unambiguously identified CKI types, we performed a systematic audit of the DBT sequences across insects with three goals: explore possible patterns in the C-terminal tail variability, determine when the NKRQK motif arose, and identify whether we may correlate these changes in DBT with additional changes in the circadian clock setup.

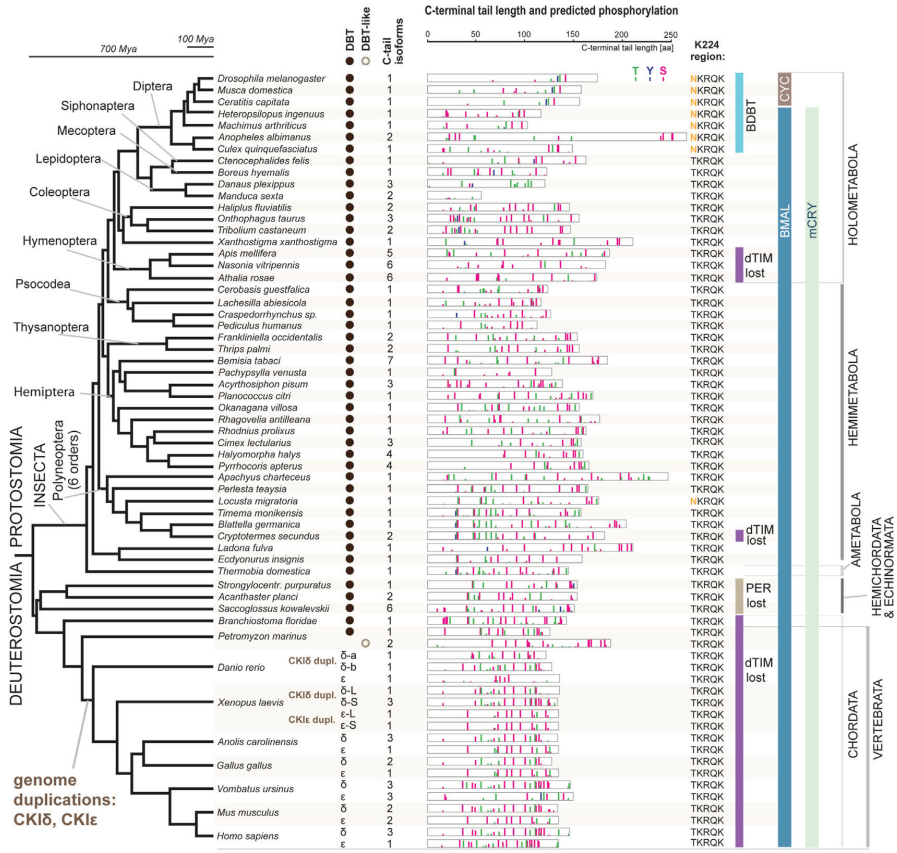


FIGURE 3
DBT and CKIδ/ε genes and proteins mapped on insect and deuterostomian phylogeny. The phylogenetic tree corresponds to a consensus of recent phylogenomic studies (Misof et al., 2014; Johnson et al., 2018; Kawahara et al., 2019; McKenna et al., 2019; Wipfler et al., 2019). Representative species are shown at the terminal nodes. The first column indicates the presence of DBT-coding genes (note a *Petromyzon*-specific gene duplication). Greek letters refer to the presence of CKIδ and CKIε; note two CKIδ paralogs in *Danio* and *Xenopus* and two CKIε paralogs in *Xenopus* (for details on CKIδ and CKIε phylogeny, see Figure 4). The second column indicates how many splicing isoforms affecting the C-terminal tail protein sequence were identified. The C-terminal tail of the longest isoform is depicted for each gene in each species (see Supplementary Figures S2–S7 for all isoforms and additional species). The color bars indicate *in silico* predicted phosphorylation patterns of threonine (T, green), tyrosine (Y, blue), and serine residues (S, pink). The bar's height refers to the predicted score between 0.5–1.0. K224 region indicates whether NKRQK or TKRQK motif was found in the region corresponding to the 220–224 position within the catalytic domain of *D. melanogaster* DBT. Major changes in the circadian clock setup are depicted: presence of CRY mammalian (mCRY) type, loss of TIMELESS-drosophila type (dTIM), loss of PERIOD (PER) (Kotwica-Rolinska et al., 2022b), and transition of BMAL (activation domain is present) to CYC (activation domain lost) (Supplementary Figure S8). DBT-interacting protein paralogs BRIDE of DBT (BDBT) was found only in Diptera (for details and BDBT phylogeny see Figure 5).

In our comprehensive analysis, we identified and further analyzed DBT sequences from 55 species representing 20 insect orders and 9 deuterostomian classes (Figure 3, Supplementary Figures S2–S7, and Supplementary Table S2). Whereas only one *dbt* gene was found in all analyzed insects, up to as many as three *dbt* paralogs were identified in the zebrafish *Danio* and four paralogs in the clawed frog *Xenopus*. In mammals, reptiles, and birds, two paralogous genes, CKIε and CKIδ, are known. Similarly, in the sea lamprey *Petromyzon marinus*, two *dbt*-like genes were discovered; however, a detailed sequence comparison indicates that these *dbt*-like genes result from lamprey-specific gene duplication (Figure 4). The CKIε/CKIδ separation is observed in sharks, rays, and fishes, and thus seems to be a result of gene duplication specific to Gnathostomata. The

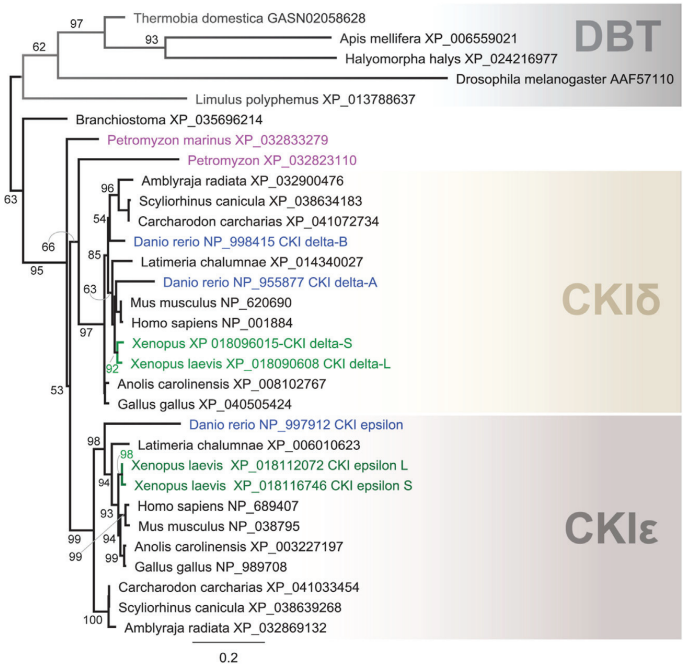


FIGURE 4
Phylogeny of DBT, CKIδ, and CKIε indicates several gene duplications in vertebrates. In the lancelet *Branchiostoma*, only one CKI gene was found to precede the δ- and ε-isoforms. The major duplication that gave rise to the δ and ε isoforms dates back to the ancestor of Gnatostomata (vertebrates with jaws), while two CKI genes in the sea lamprey *Petromyzon marinus* resulted from *Petromyzon*-specific gene duplication. CKIδ was duplicated in the zebrafish *Danio rerio*, resulting in the so-called CKIδ-A and CKIδ-B. In the African clawed frog *Xenopus laevis*, a large genome duplication resulted in two CKIδ (L and S) and CKIε (L and S) genes. DBT sequences from protostomian representatives (the firebrat *Thermobia domestica*, the honey bee *Apis mellifera*, the marmorated sting bud *Halyomorpha halys*, the fruit fly *Drosophila melanogaster*, and the horseshoe crab *Limulus polyphemus*) were used as outgroups. The tree was inferred using RAxML maximum likelihood of 31 protein sequences using Geneious 11 software (Biomatters). Bootstrap support was calculated using 100 replications.

second duplication of CKIδ led to two CKIδ genes present in the zebrafish *Danio rerio* and probably a similar but independent (*Xenopus*-specific) duplication happened in the ancestor of the clawed frog *Xenopus laevis*. In addition to gene duplications and quadruplications, a various number of protein isoforms can be produced in some organisms from individual genes as a result of alternative splicing (see the C-terminal tail analysis below).

NKRQK region and bride of DBT

The NKRQK motif (positions 220–224 in *Drosophila* DBT) within the catalytic domain distinguishing *D. melanogaster* DBT from the mouse homologs (Figure 1) was identified in all analyzed dipteran insects (Figure 3). Apart from the desert locust *Locusta migratoria* (the sequence was confirmed by Sanger sequencing and only one *dbt* gene was identified in the genome), all non-dipteran species contain the TKRQK motif. Therefore, we compared the presence of NKRQK to known changes in the circadian clock setup, such as the presence of mCRY, loss of dTIM (Kotwica-Rolinska et al., 2022a), and transformation of BMAL to CYC (Meireles-Filho et al., 2006). The transition of BMAL, a transcription factor with a transactivation domain, to CYC, a transcription factor which lacks the transactivation domain, was identified exclusively in Cyclorrhapha, a subset of Diptera (Supplementary Figure S8). However, this change perfectly corresponds to the loss of mCRY and does not agree with the presence of NKRQK (Figure 3).

Since none of the known changes in the clock setup correlated with the presence of the NKRQK motif in DBT, we analyzed the evolution of BDBT, a non-canonical FK506-binding protein interacting with DBT in *Drosophila* (Fan et al., 2013). First, we performed a phylogenetic analysis of available known FK506-binding proteins. The unrooted phylogenetic tree in

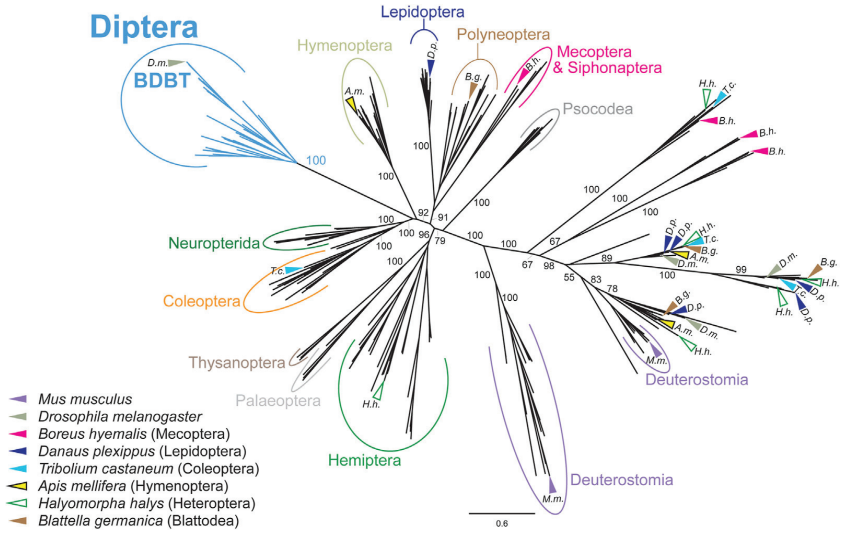


FIGURE 5
Phylogeny of FK506-binding proteins revealed a clear separation of dipteran BRIDE of DBT (BDBT) from all remaining clusters. Major insect orders are highlighted. Arrows indicate the position of sequences from representative species. FK506-binding proteins from Mecoptera and Siphonaptera do branch far away from Diptera. The tree was inferred using RAxML maximum likelihood of 280 protein sequences (final GAMMA-based score of the best tree −104683.7791) using Geneious 11 software (Biomatters). Bootstrap support was calculated using 100 replications.

Figure 5 (and the full tree version in Supplementary Figure S9) represents how various FK506-binding proteins evolved over time. Notably, dipteran BDBTs form a clear cluster that is separated from all remaining proteins. Furthermore, FK-506 binding proteins from Mecoptera (Scorpionflies) and Siphonaptera (Fleas), the closest relatives of Diptera, do not cluster with BDBT. Therefore, BDBT has been so heavily modified in Diptera that we cannot reliably identify the corresponding BDBT in any non-dipteran insect. Interestingly, in Diptera, the rise of the BDBT gene correlates with the transition from TKRQK to NKRQK motifs. The only other occurrence of the NKRQK motif was observed in *Locusta* (Orthoptera), whereas the sister polyneopteran lineages (termites, roaches, phasmids, Mantophasmatodea) contain TKRQK. However, FK-506 binding proteins of all polyneopteran lineages (including Orthoptera) branch together independently of TKRQK to NKRQK motifs.

The C-terminal tail of CKI

The well-established impact of the C-terminal tail on the priming capacity of CKIδ splice isoforms in mice (Narasimamurthy and Virshup, 2021) prompted us to explore the C-terminal tail variability in the identified DBT/CKI dataset. The *in silico* predicted phosphorylation pattern was depicted for probabilities >0.5 (Figure 3 and Supplementary Figures S2–S7). When compared to the kinase domain, the C-terminal tails are the most variable parts of the proteins. Somewhat similar phosphorylation patterns and comparable lengths are found among C-terminal tails in vertebrates. In insects, however, the length and sequence of the C-terminal tails are remarkably variable. The shortest tails were identified in Lepidoptera (in several species only around 50 amino acids), whereas the longest tail in the *Anopheles* mosquito exceeded 250 amino acids (Figure 3). Putative phosphorylation was more prevalent in the C part of the C-terminal tail; however, the predicted phosphorylation patterns were quite variable in insects.

Alternative splicing of the C-terminal tail

Alternative splicing of mouse *CKIδ* transcripts affects the biochemical properties of resulting CKIδ protein isoforms. In all analyzed vertebrate species, CKIδ was alternatively spliced with impact on the predicted phosphorylation pattern in the terminal part of the C-terminal tail. In contrast, CKIε was alternatively spliced only in a few vertebrate species (Supplementary Figure S7). Alternative splicing was detected in *dbt* of many insects, including the mosquito *Anopheles albimanus* (Diptera), all

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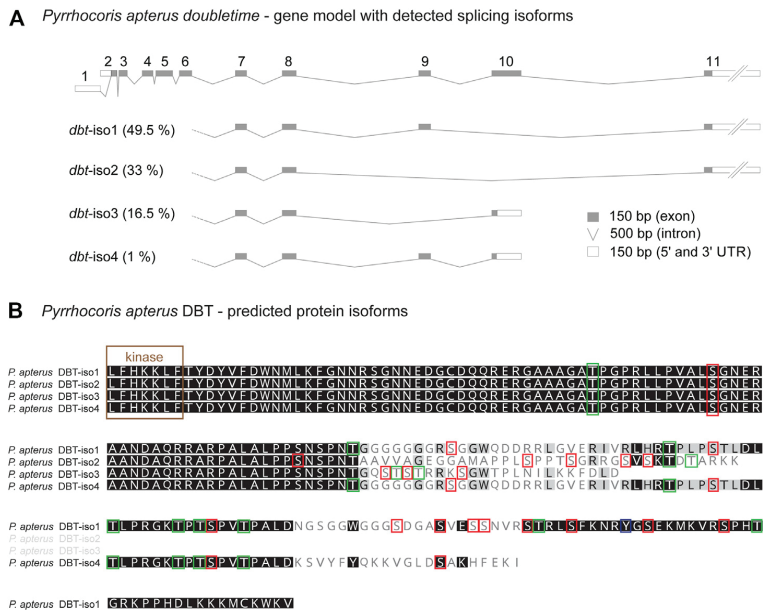

FIGURE 6
Alternative splicing of *doubletime* (*dbt*) gene in the linden bug *Pyrrhocoris apterus*. **(A)** A gene model illustrating the length of all *dbt* exons and introns. Two alternative transcription starts and splicing at the 5′end (exon 1 or 2, respectively) do not influence the predicted protein coding sequence. Alternative skipping of exons 9 and/or 10 results in four isoforms (*dbt*-iso1, 2, 3, 4). The expression level of each *dbt* isoform mRNA in the brain was detected from the Oxford nanopore transcriptome and depicted as % contribution to all *dbt* molecules (100%—all isoforms). **(B)** Protein alignment of the C-terminal tail illustrating the isoform-specific and variable regions (the more conserved residue the darker the color). Color rectangles refer to the theoretical prediction of phosphorylation pattern (>0.5; NetPhos-3.1) for serine (S), threonine (T), and tyrosine (Y).

analyzed beetles (Coleoptera), all analyzed butterflies/moths (Lepidoptera), all analyzed hymenopteran species (the honey bee *Apis mellifera*, the jewel wasp *Nasonia vitripennis*, and the turnip sawfly *Athalia rosae*), the pea aphid (*Acyrthosiphon pisum*), both analyzed species of Thysanoptera (the western flower thrips Frankliniella occidentalis and the melon thrips *Thrips palmi*), the silverleaf whitefly *Bemisia tabaci*, true bugs (Heteroptera: the water strider *Rhagovelia antilleana*, the kissing bug *Rhodnius prolixus*, the common bed bug *Cimex lectularius*, the brown marmorated stink bug *Halyomorpha halys*, and the linden bug *Pyrrhocoris apterus*), and the drywood termite *Cryptotermes secundus* (Supplementary Figures S3–S5). As a representative of the true bugs, having access to the linden bug *P. apterus* brain transcriptome obtained by Oxford Nanopore Technology, we analyzed the presence and abundance of all four identified *dbt* isoforms (Figure 6). The three most abundant isoforms comprising 99% of *dbt* transcripts encode proteins with predicted altered phosphorylation patterns. Thus, alternative splicing of *dbt* might serve as a regulatory step influencing and modulating the properties of DBT in some insects. In flies including *Drosophila*, however, *dbt* is an intronless gene. A similar intronless gene organization might exist in other insect species. However, the identification of only one transcript variant should be interpreted with caution. The reliable decision whether *dbt* gene contains introns requires a good non-fragmented genome assembly, ideally with well-annotated gene models. On the other hand, the identification of only one *dbt* isoform in the transcriptome of a particular species may reflect only shallow sequencing or might be affected by transcript assembly and post-sequencing processing.

The C-terminal tail of DBT in diptera

The absence of introns in *dbt* genes of flies and the remarkable diversity of the C-terminal tail among flies and mosquitoes motivated a detailed comparison of this part of DBT in Diptera. Protein alignment of 15 species representing major dipteran lineages revealed a conserved region in the C-terminal tail, where a short 12 amino acid motif is identified in all dipteran species, and an even longer motif is shared among Cyclorrhapha (Figure 7). This conserved motif

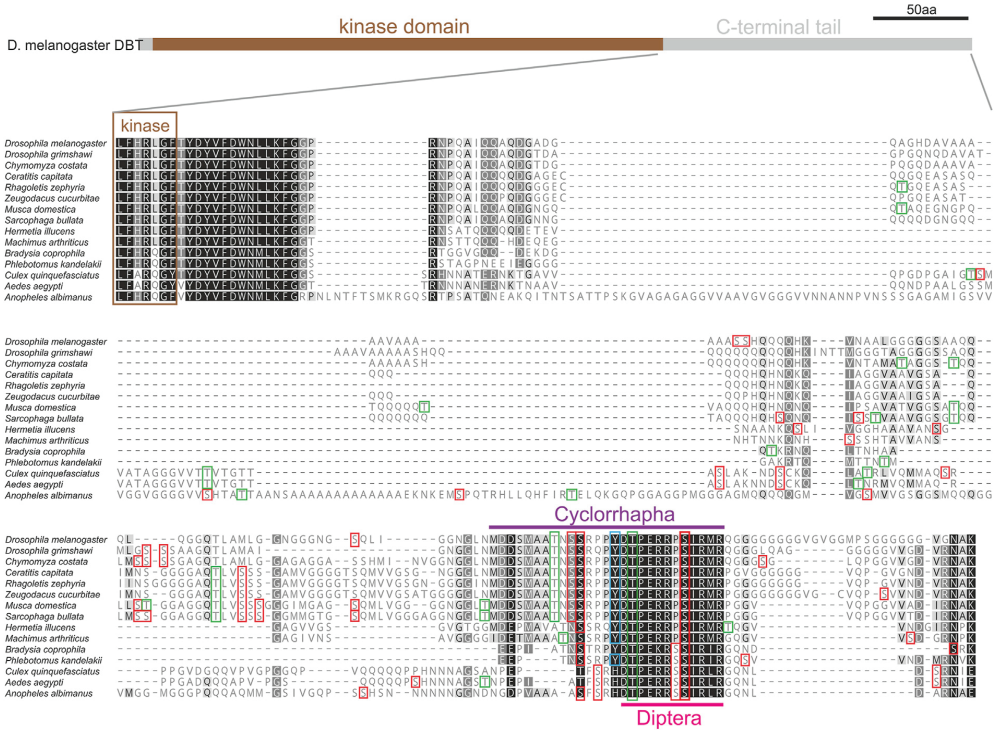

FIGURE 7
C-terminal tail of DBT in 15 representative species of Diptera. Protein alignment points to variable and conserved regions in the protein sequence (the more conserved the darker the color; a hyphen corresponds to a gap in the alignment). Color rectangles refer to the theoretical prediction of phosphorylation pattern (>0.5, NetPhos-3.1) for serin (S), threonine (T), and tyrosine (Y). The C-terminal tail contains a motif conserved in Diptera (light purple), which can be further extended in Cyclorhapha (Deep Purple).

contains residues with high scores predicting their phosphorylation.

Functional experiments in *Drosophila*

To test whether the identified sequence motifs in DBT have an impact on the function of the circadian clock *in vivo*, we applied reverse genetic tools to the fruit fly *D. melanogaster* and focused on two regions: 1) residue K224, which was established as important for temperature compensation in mammalian CKIδ (Shinohara et al., 2017), and which is also part of NKRQK motif (Figure 3), and 2) the C-terminal tail (Figure 7).

Three different C-terminal tail mutants were created, encompassing or bordering the conserved C-terminal tail domain (Figures 8A, B). All of them are homozygous viable and displayed only very mild, yet significant circadian phenotypes. The deletion of amino acids downstream of position 370, that is the part comprising conserved cyclorrhaphan and dipteran motifs, slightly extended τ when compared to controls (Figures 8C, D and Supplementary Table S4). Deletions of the very end of the C-terminal tail and frameshift (Δ411–440) and a 15-bp in-frame deletion upstream of conserved motifs (Δ366–370) mildly shortened τ (Figures 8E, F and Supplementary Table S4).

The basic lysine residue K224 was replaced by the acidic residues aspartic acid (D) or glutamic acid (E), respectively. In both cases, homozygous mutants were not viable, thus, heterozygous flies were analyzed. In both mutants, τ was significantly shorter compared to control flies. Moreover, these two mutants differ in their temperature compensation (Figures 8G–J). The K224D heterozygotes displayed a τ of ~20 h at 18°C and lengthening of τ up to 22 h at higher temperatures (Q_{10} = 0.94) (Figures 8H, J). The K224E heterozygotes displayed an approximately 5-h faster clock compared to the wild type (τ ~ 19) at all three tested

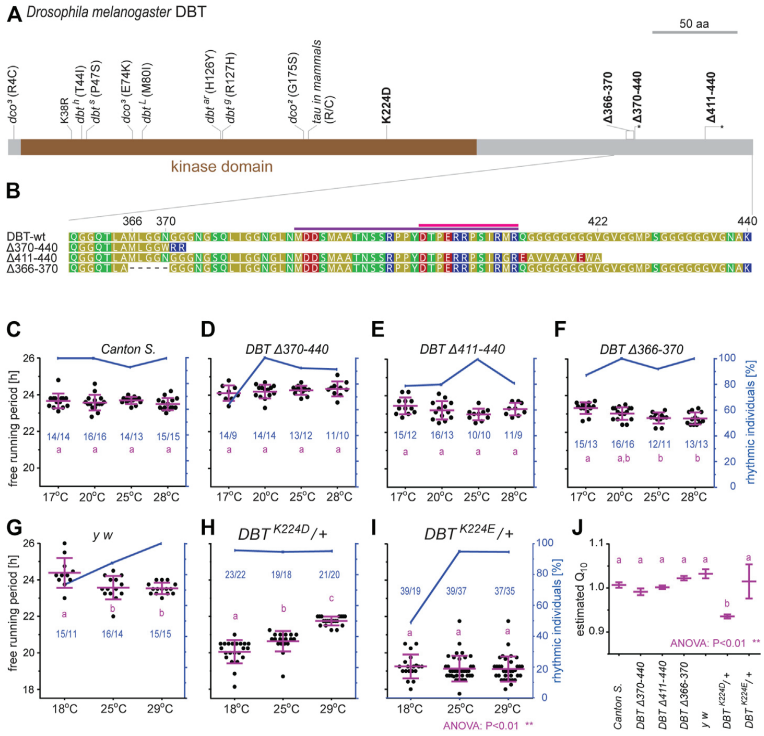

FIGURE 8
Functional analysis of new *dbt* mutants created in *D. melanogaster*. **(A)** A schematic depiction of DBT protein with highlighted mutations; here-created mutants are in bold, the asterisk in the scheme refers to a premature protein termination due to a frameshift. **(B)** Detail of the C-terminal tail in the wild type (wt) and three mutant lines. The dipteran and cyclorrhaphan motifs are highlighted by light and deep purple, respectively. **(C–I)** Circadian clock phenotype [each dot in panels **(C–I)** represents the free-running period tau, τ, of individual male flies] was recorded for 10 days in DD at the specified temperature. Blue dots connected with a blue line indicate the percentage of rhythmic individuals at a particular temperature. A scale indicating the percentage rhythmicity is on the right y-axis, the blue numbers in the chart represented $n_{total}/n_{rhythmic}$. **(J)** The temperature compensation depicted as Q_{10} was calculated from data presented in **(C–I)**. Magenta bars represent means ± SEM.

temperatures and showed reduced rhythmicity at 18°C (Figures 8I, J). The shorter τ corresponded to earlier evening activity peaks in LD regimes in both mutant lines, K224D (Supplementary Figure S11) and K224E (Supplementary Figure S12), when compared to control *y w* flies (Supplementary Figure S10).

Discussion

Recent remarkable progress in the genome and transcriptome sequencing allowed us to retrieve *dbt/CKI* genes from all major insect orders and from representative vertebrate lineages, and perform their sequence comparison. The available data point to several mutually independent CKI duplications observed in the deuterostomian lineage. Our analysis suggests that two *dbt*-homologous genes identified in *P. marinus* are the result of a *Petromyzon*-/lamprey-specific duplication, whereas duplication leading to the rise of *epsilon* and *delta* isoforms of CKI is dated to the common ancestor of Gnatohostomata. The complexity of the circadian clock setup in *Danio* and *Xenopus* has been shaped by independent genome duplications. In *Danio*, this gene duplication reflects complexity identified in many teleost species (Garcia-Concejo and Larhammar, 2021), however, in *Xenopus*, we see the outcome of a very recent species-limited genome duplication (Uno et al., 2013).

In addition to gene duplications, further diversity of CKIδ/ε is achieved *via* alternative splicing. Notably, CKIδ is alternatively spliced in all tetrapods (amphibia, reptiles, birds, and mammals), where the splicing influences the

C-terminal tail of the protein including its putative phosphorylation pattern. In *Danio*, only one splice variant was identified for each CKIδ paralogue, thus, the protein diversity of CKIδ might be achieved by independent genes in this species.

The diversity of DBT sequences in insects is remarkable, and we suggest that the role of alternative splicing will most likely differ among various insect lineages. As we show in the linden bug *P. apterus*, not only are multiple splicing isoforms encoded by the *dbt* gene but three of them are expressed in the brain. Since *dbt* silencing results in a remarkable extension of τ in *P. apterus* (Kotwica-Rolinska et al., 2022a), functional tests of each splicing isoform would be an interesting research direction. Unfortunately, the isoform-specific exon 9 is only 159 nt long. Therefore, we are reaching technical limitations of ds RNA mediated interference, even though a 288 bp long dsRNA was successfully used to knock down isoform-specific transcripts (Bajgar et al., 2013). An alternative approach could utilize gene editing, a method established and used for circadian research in *P. apterus* (Kotwica-Rolinska et al., 2019; Kotwica-Rolinska et al., 2022b).

In some insects, however, no alternative splicing was detected and in certain lineages, such as flies, the *dbt* gene is intronless. Interestingly, our comprehensive analysis revealed three new *Drosophila* genus-specific CKI genes. To our knowledge, the role and function of these casein kinases are not established. Given the participation of both DBT and CKIα in the fruit fly circadian clock (Lam et al., 2018), one cannot rule out the involvement of here-identified CKI genes in the circadian clock. Although this is entirely speculative, the combination of multiple kinases encoded by independent genes would provide an alternative source to the isoform repertoire produced by alternative splicing from an individual gene in some other species.

Functional analysis of the C-terminal tail in *D. melanogaster* revealed negligible effects on rhythmicity or changes in τ. However, only three simple deletion mutants were created here, thus, a full evaluation of the role for the C-terminal tail in DBT is rather premature. The second set of mutants, K224 modifications, resulted in homozygous lethality, similar to known strong *dbt/dco* alleles disrupting the developmental function of CK1 during the pupal stage (Price et al., 1998; Zilian et al., 1999). The equivalent mammalian mutations are not homozygous lethal, presumably because CK1δ and CK1ε are able to complement each other. Heterozygous mutant K224D/+ and K224E/+ flies produced profound shortenings of τ, almost identical to homozygous CK1δ K224D mutant mice (Shinohara et al., 2017, Figures 8G–I). Acidic K224 substitutions presumably bypass the phospho relay embedded in the per^S serine cluster (FASP serine cluster in mPER2), and immediately phosphorylate the respective serine in the PER phosphodegron. This is because in mammalian CK1, K224 (together with R178) forms an anion-binding site required for phosphorylation of primed substrates (Narasimamurthy and Virshup, 2021). In flies and mammals, phosphorylation of the per^S/FASP serine cluster delays PER degradation by preventing premature phosphorylation at the phosphodegron site, which would lead to rapid PER turnover (phosphoswitch). However, phosphorylation of multiple serine residues within the per^S/FASP cluster by DBT/CK1 requires a priming phosphorylation at a particular serine within each cluster. Binding of DBT/CK1 to this primed substrate requires the basic anion binding pocket formed by R178 and K224, which most likely can not form in acidic K224D and K224E mutants. Therefore, the short periods observed in K224D, K224E, and R178 (= hamster *tau* mutant: Ralph and Menaker, 1988; Lowrey et al., 2000) are presumably the consequence of impaired per^S/FASP region phosphorylation and the resulting acceleration of phosphorylation at the phosphodegron site. Moreover, the K224D mutation affects temperature compensation, both in flies and mouse organ cultures. However, the temperature-dependent lengthening of τ associated with the K224D mutation in flies is opposite to what was detected for *mPer2-luc* expression in suprachiasmatic nucleus (SCN) slices of homozygous K224D mice, which showed a shortening of τ with increasing temperature (Shinohara et al., 2017). The opposite temperature compensation phenotypes of K224D in flies and mice, as well as the lack of temperature-dependent period lengthening in the very similar K224E mutants, suggest that K224D overcompensation in flies is not simply caused by potential thermal instability of the K224D protein. Moreover, temperature overcompensation is not generally linked to decreased protein stability at higher temperatures (Giesecke et al., 2021). The phenotypic differences between the fly K224D and K224E mutants are surprising (temperature overcompensation in K224D and reduced rhythmicity at 18°C in K224E, Figure 8). These differences demonstrate that the two replacements have different consequences (apart from both shortening the free-running period), even though both introduce an acidic residue. Although very similar in structure, glutamic acid is slightly larger compared to aspartic acid, which may influence substrate binding in a temperature-dependent manner. In addition, the presence of an Asparagine (N) at position 220 in fly DBT, as opposed to a Threonine (T) at this position in CKIδ, might contribute to the opposite temperature compensation phenotypes of K224D mutants in flies and mice. Replacing the fly Asparagine (N220) with a Threonine would be interesting, not only in the light of temperature compensation differences, but also in the context of recently published autophosphorylation of Threonine, which is the preferred amino acid in corresponding positions in the majority of homologous kinases (Cullati et al., 2022). Secondly, the possible interaction with BDBT in the fruitfly might be an interesting and experimentally testable explanation for the altered temperature compensation in K224D. On the other

hand, the NKRQK motif arose independently in *Locusta* and other orthopteran species and, given the diversity of FK-506 binding proteins, can hardly be interpreted as BDBT-dependent modification. Moreover, given the broad range of targets phosphorylated by DBT and even its non-catalytic role in the circadian clock (Yu et al., 2009), the mechanistic model might be quite complex.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

DD and RS designed the study. NT, AG, OB, and JM performed *Drosophila* experiments. DD run all phylogenetic analyses with help of VS, who also established the *P. apterus* dbt gene model. DD wrote the manuscript with input from NT and RS.

Funding

The work, DD, VS, and NT were supported by the Czech Science Foundation (GACR) grant number 22-10088S. AG and RS were supported by the Deutsche Forschungsgemeinschaft grant STA 421/7-1. ONT sequencing experiments were supported from European Research Council (ERC) under the European Union's Horizon 2020 Program Grant Agreement 726049. We acknowledge support from the Open Access Publishing Fund of University of Münster.

Acknowledgments

We thank Dr. Samarjeet Singh for advice on transgenesis and CRISPR/CAS9 mutagenesis, and Bulah C. Wu for technical assistance with ONT genome and transcriptome sequencing and assembly. For ONT processing we thank Computational resources which were supplied by the project "e-Infrastruktura CZ" (e-INFRA CZ LM2018140) supported by the Ministry of Education, Youth and Sports of the Czech Republic.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1062632/full#supplementary-material

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Supplementary text describing circadian clock gene terminology

***Dosophila-type timeless* (*d-tim*), dTIMELESS (dTIM), (CG3234)**
is used for the *Drosophila*-type *timeless* gene/transcript (in italics) and protein (in capitals) in this publication. However, the terminology varied in history:
Simple abbreviations ***tim*** and **TIM** were used in the early studies (Ashmore et al., 2003; Myers et al 1995; Sehgal et al., 1994; Stanewsky et al., 2002; Hunter-Ensor et al 1996; and many others). With the discovery of an alternative transcription start, ***ls-tim***, ***s-tim*** and **L-TIM**, **S-TIM** were introduced (Rosato et al., 1997; Tauber et al., 2007; with Peschel et al., 2009, using L-Tim, S-Tim and Tim for protein). Additional splicing variants were described and labeled ***tim-sc***, ***tim-M***, ***tim-cold***, and ***tim-L*** (Martin Andagua et al., 2019, Boothroyd et al. 2007; Foley et al. 2017). In mammals, a homologous gene *timeless* (*tim*) was discovered in the late 90s (Zylka et al., 1998). However, a few years later it turned out that *Drosophila* and other insect species also possess a homolog of this mammalian ***tim.*** Thus, *Drosophila* has two *timeless* genes (two paralogs): the *Drosophila* type and the mammalian type. The first (CG3234) was sometimes labeled as ***tim1*** (**TIM1** for the protein) and the latter as ***tim2*** (**TIM2**) (Benna et al. 2000, 2010; CG7855). However, Reppert's laboratory published a parallel terminology with **TIM** for the *Drosophila*-type and **TIMEOUT** for the mammalian type in *Drosophila*, **mTIM** for the mouse protein, and ***C.e.*** **TIM-1** for protein from *Caenorhabditis elegans* (Gotter et al., 2000). The latter one is rather unfortunate because the *C. elegans* protein is actually branching with the mammalian types (labeled as TIM2 by Benna). Later, Engelen used **dTIM** and **dTIM-2 or dTIM2** for the two *Drosophila* paralogs; **s-TIM** and **l-TIM** for alternative transcription starts, whereas **TIM** served as abbreviation for the mammalian protein (Engelen et al., 2013). In 2017, Bazalova et al. published a comparative study between fruit fly and house fly in which the *Drosophila*-type *timeless* was analyzed and described *Drosophila tim* as ***Dm_tim*** and *Musca domestica* as ***Md_tim***. A recent study, highlighting the loss of several circadian genes across bilateria and insects, used TIMELESS-d (TIM-d) for light sensitive TIM (or TIM1) and TIMELESS-m (TIM-m) for light-insensitive and mammalian-type TIM (or TIM2, timeout) (Kotwica-Rolinska et al., 2022).

***Dosophila-type cryptochrome* (*d-cry*), dCRYPTOCHROME (dCRY)** is used for the *Drosophila*-type *cryptochrome* gene/transcript (in italics) and protein (in capitals) in this publication. Several studies also used **CRY1** for the *Drosophila*-type and **CRY2** for the mammalian-type, although the latter is also present in some but not all insects (Zhu et al., 2006; Yuan et al., 2007). Importantly, mammals have two CRYPTOCHROME paralogs, historically labeled as **CRY1** and **CRY2**, yet both belong to the mammalian type. Furthermore, the name 'evolved' due to functional variance between mammalian and *Drosophila* CRY: the latter often referred to as dCRY (Vaidya et al., 2013; Baik et. al. 2019; Chandrasekaran et al., 2021 and others) and mammalian CRY referred to as mCRY1 and mCRY2 (e.g., Oster et al., 2003; Collins et al.,2006). Recent biochemical and the structural studies of *Drosophila* CRYPTOCHROME have been using different alternative versions such as DmCry (Arthaut et al., 2017), *Dm*Cry (Berntsson et al., 2019; Einholz et al., 2021) and DmCRY (Kutta et al., 2018). Furthermore, some of the earlier studies used *dcry* or *DCry* for the gene, and DCRY for the protein, respectively (Ishikawa et al., 1999 Egan et al., 1999). A recent phylogenetic study adapts the terminology such as PCRY

(plant-specific), ACRY (cnidirian-specific), DCRY (*Drosophila*-type) and MCRY (mammalian-type), all used for protein (Deppisch et al., 2022). Studies on non-model insects reported the presence of CRY which is sequentially and functionally more conserved to the mammalian version of CRY, hence distinguishing both as *Drosophila*-type CRYPTOCHROME (CRY-d) and mammalian-type CRYPTOCHROME (CRY-m) (Rubin et al., 2006; Ikeno et al., 2008, 2011a,b; Ingram et al., 2012, Kotwica-Rolinska et al., 2022).

***doubletime* (*dbt*)**, **DOUBLETIME** was discovered as a circadian clock protein in 1998 in *Drosophila* (Price et al., 1998; Kloss et al., 1998). One year later, it turned out that the *dbt* gene is identical to ***disc overgrown*, *dco*,** a gene that was identified for its developmental role (Zilian et al., 1999). Mammalian literature discriminates between **α**, **γ**, **ε**, and **δ** isoforms of the casein kinase I proteins (**CKI** or **CK1**), even though each of them is encoded by a completely different gene (and there are even three paralogous γ genes in mammals) (Zhang et al., 1996; Green and Bennett, 1998; Gross and Anderson, 1998; Rivers et al., 1998; Knippschild et al., 2005; Zheng et al., 2014).

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Number of families	**1**							
Number of comparisons per family	3							
Alpha	0.05							
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
CS vs. DBT ?370-440	-0.6236	-0.9589 to -0.2883	Yes	***	0.0008			
CS vs. DBT ?366-370	0.6424	0.3134 to 0.9714	Yes	***	0.0006			
CS vs. DBT ?411-440	0.3824	0.04650 to 0.7182	Yes	*	0.0256			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
CS vs. DBT ?370-440	23.60	24.23	-0.6236	0.1244	58	45	5.014	12
CS vs. DBT ?366-370	23.60	22.96	0.6424	0.1221	58	55	5.263	12
CS vs. DBT ?411-440	23.60	23.22	0.3824	0.1246	58	44	3.069	12

Number of families	**1**							
Number of comparisons per family	2							
Alpha	0.05							
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value			
y w vs. K224D	3.011	1.693 to 4.329	Yes	**	0.0011			
y w vs. K224E	4.656	3.343 to 5.968	Yes	***	0.0001			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
y w vs. K224D	23.82	20.81	3.011	0.4673	40	60	6.445	6
y w vs. K224E	23.82	19.17	4.656	0.4653	40	91	10.01	6

Primer name	Sequence	Used for
dbtBMHRF	Gatggtcgactctagaagtgatcgcgagacgggt	Outside primer for amplifying 5' homology region
dbtBMHRR	gatggtcgacaagcttGTATGGATTGGATTCGCTAGAGC	Outside primer for amplifying 3' homology region
dbtPamT1mutF	CGCCTTGCCATGGCAAGGCTTAAAGGCAGCCAACA	Introduces Pam site mutation for gRNA target
dbtPamT1mutR	TTGCCATGGCAAGGCGCCCAGATTGAAGTACA	Introduces Pam site mutation for gRNA target
dbtSalIF	GAGAAGAAGCTGTCGACCTCGATTGTGGTGCTG	Introduces SalI site
dbtSalIR	GTCGACAGCTTCTTCTCCGAGATCCTCT	Introduces SalI site
dbtK224DF	CCAACAAGAGGCAAGACTACGAGAGGATCTCGGAGAA	Introduces K224D mutation
dbtK224DR	GTCTTGCCTCTTGTTGGCTGCCTTTAAGCC	Introduces K224D mutation
dbtK224EF	CCAACAAGAGGCAAGAGTACGAGAGGATCTCGGAGAA	Introduces K224E mutation
dbtK224ER	CTCTTGCCTCTTGTTGGCTGCCTTTAAGCC	Introduces K224E mutation
SeqDbtF	CATCGCGACATCAAGCCGGATAAC	Sequences modified dbt region
SeqDbtR	TGGAGTTGCTGTTGCGCTGCACTGCC	Sequences modified dbt region
dbtscreenF1	CATCGCGACATCAAGCCGGATAACTTCC	Molecular screen of transformants via SalI site
dbtscreenR	TGCCGCATCCGTATCGAAGGCCGACGC	Molecular screen of transformants via SalI site
DbtgRNAT1F	GTCGGTGGCAGTAATCGGGACGC	gRNA target
DbtgRNAT1R	AAACGCGTCCCGATTACTGCCACC	gRNA target

Species/group	Gene	Protein isoforms	note
Drosophila melanogaster Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha; Drosophilidae	1	AAF57110	1 protein isoform; no alternative splicing possible (intronless gene)
Drosophila grimshawi Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha; Drosophilidae	1	GH14346	1 protein isoform (identified in TSA)
Chymomyza costata Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha; Drosophilidae	1	JAECWU010000002	1 protein isoform; identified in genome no alternative splicing possible (intronless gene)
Ceratitis capitata Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha, Tephritidae	1	XP_004523403	1 protein isoform;
Rhagoletis zephyria Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha, Tephritidae	1	XP_017479213	1 protein isoform (identified in TSA)
Zeugodacus cucurbitae Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha, Tephritidae	1	XP_011186061	1 protein isoform (identified in TSA)
Musca domestica Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha, Muscidae	1	XP_019890952	1 protein isoform;
Sarcophaga bullata Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha, Sarcophagidae	1	TMW47958	1 protein isoform (identified in TSA)
Heteropsilopus ingenuus Insecta; Holometabola; Diptera; Brachycera; Empidoidea; Dolichopodidae;	1	Heteropsilopus ingenuus GCGO01028505	1 protein isoform (identified in TSA)
Hermetia illucens XP_037913096 Insecta; Holometabola; Diptera; Brachycera; Stratiomyomorpha; Stratiomyidae	1	XP_037913096	1 protein isoform (identified in TSA)
Machimus arthriticus Insecta; Holometabola; Diptera; Brachycera; Asiloidea; Asilidae	1	GFZQ01005943	1 protein isoform (identified in TSA)
Bradysia coprophila Insecta; Holometabola; Diptera; Nematocera; Sciaroidea; Sciaridae;	1	XP_037029789	1 protein isoform (identified in TSA)
Phlebotomus kandelakii Insecta; Holometabola; Diptera; Nematocera; Psychodoidea; Psychodidae;	1	NBJ59035	1 protein isoform (identified in TSA)
Anopheles albimatus Insecta; Holometabola; Diptera; Nematocera; Culicidae	1	XP_035774255 XP_035774260	2 protein isoforms
Aedes aegypti Insecta; Holometabola; Diptera; Nematocera; Culicidae;	1	XP_021703386	1 protein isoform
Culex quinquefasciatus	1	XP_038108204	1 protein isoform

Insecta; Holometabola; Diptera; Nematocera; Culicidae			
Ctenocephalides felis Insecta; Holometabola; Siphonaptera; Pulicidae	1	XP_026472425	1 protein isoform
Boreus hyemalis Insecta; Holometabola; Mecoptera	1	GAYK02023579	1 protein isoform
Danaus plexippus Insecta; Holometabola; Lepidoptera; Ditrysia; Nymphalidae	1	OWR42709 XP_032518479 XP_032518488	3 protein isoforms
Manduca Insecta; Holometabola; Lepidoptera; Ditrysia; Bombycoidea; Sphingidae;	1	KAG6442350 XP_030035925	2 protein isoforms
Haliplus fluviatilis Insecta; Holometabola; Coleoptera; Adephaga; Haliplidae		GDMW01032753 GDMW01032754	2 protein isoforms
Onthophagus taurus Insecta; Holometabola; Coleoptera; Polyphaga; Scarabaeidae		XP_022916674 XP_022916672 XP_022916673	3 protein isoforms
Tribolium castaneum Insecta; Holometabola; Coleoptera; Polyphaga; Tenebrionidae		XP_015838159 XP_015838158	3 protein isoforms
Xanthostigma xanthostigma Insecta; Holometabola; Raphidioptera; Raphidiidae		GAUI02055818	1 protein isoform (identified in TSA)
Apis mellifera Insecta; Holometabola; Hymenoptera; Apocrita; Aculeata; Apoidea; Apidae		XP_006559020 XP_006559021 XP_006559017 XP_026299552 XP_006559019	5 protein isoforms
Nasonia vitripennis Insecta; Holometabola; Hymenoptera; Apocrita; Parasitoidea; Chalcidoidea; Pteromalidae		XP_016838712 XP_031781923 XP_031781924 XP_016838713 XP_016838714 XP_031781922	6 protein isoforms
Athalia rosae Insecta; Holometabola; Hymenoptera; Tenthredinoidea; Tenthredinidae		XP_020712524 XP_012269380 XP_020712523 XP_020712525 XP_012269382 XP_012269381	6 protein isoforms
Cerobasis guestfalica Insecta; Paraneoptera; Psocodea; Psocoptera, Trogiidae		GDEA01034316	1 protein isoform (identified in TSA)
Lachesilla abiesicila Insecta; Paraneoptera; Psocodea; Psocoptera, Lachesillidae		GDEL01015526	1 protein isoform (identified in TSA)
Craspedorrhynchus sp. Insecta; Paraneoptera; Psocodea; Phthiraptera; Philopteridae		GCWN01029268	1 protein isoform (identified in TSA)
Pediculus humanus Insecta; Paraneoptera; Psocodea; Phthiraptera; Pediculidae		XP_002427537	1 protein isoform
Frankliniella occidentalis		XP_026279829	2 protein isoforms

Insecta; Paraneoptera; Thysanoptera; Thripidae		XP_026279830	
Thrips palmi Insecta; Paraneoptera; Thysanoptera; Thripidae		XP_034241879 XP_034241880	2 protein isoforms
Bemisia tabaci Insecta; Paraneoptera; Hemiptera; Sternorrhyncha; Aleyrodoidea; Aleyrodidae		XP_018907319 XP_018907318 XP_018907320 XP_018907321 XP_018907315 XP_018907316 XP_018907317	7 protein isoforms
Pachypsylla venusta Insecta; Paraneoptera; Hemiptera; Sternorrhyncha; Psylloidea; Aphalaridae		GAOP01112552	1 protein isoforms
Acyrthosiphon pisum Insecta; Paraneoptera; Hemiptera; Sternorrhyncha; Aphidomorpha; Aphididae		XP_008186371 XP_001951697 XP_008186372	1 protein isoform
Planococcus citri Insecta; Paraneoptera; Hemiptera; Sternorrhyncha; Aphidiformes; Coccoidea;		GAXF02008825	1 protein isoform (identified in TSA)
Okanagana villosa Insecta; Hemiptera; Euhemiptera; Cicadoidea; Okanagana		GAWQ02049094	1 protein isoform (identified in TSA)
Rhagovelia antilleana Insecta; Paraneoptera; Hemiptera; Heteroptera; Gerromorpha		GFOS01047006	1 protein isoform
Rhodnius prolixus Insecta; Paraneoptera; Hemiptera; Heteroptera; Cimicomorpha;		GECK01038739	1 protein isoform
Cimex lectularius Insecta; Paraneoptera; Hemiptera; Heteroptera; Cimicomorpha;		XP_014260368 XP_014260727 XP_014260607	3 protein isoforms
Halyomorpha halys Insecta; Paraneoptera; Hemiptera; Heteroptera; Pentatomomorpha;		XP_014274818 XP_024216977 XP_024216975 XP_024216976	4 protein isoforms
Pyrrhocoris apterus Insecta; Paraneoptera; Hemiptera; Heteroptera; Pentatomorpha;		iso1 OP575299 iso2 OP575300 iso3 OP575301 iso4 OP575302	4 protein isoforms
Apachyus charteceus Insecta; Polyneoptera; Dermaptera;		GAUW02029097	1 protein isoform (identified in TSA)
Perlesta teaysia Insecta; Polyneoptera; Plecoptera; Perlesta		GHRG01076916	1 protein isoform (identified in TSA)
Locusta migratoria Insecta; Polyneoptera; Orthoptera;		GETS01026013	1 protein isoform (identified in TSA)
Timema monikensis Insecta; Polyneoptera; Phasmatodea		CAD7427940	1 protein isoform
Blattella germanica Insecta; Polyneoptera; Blattodea; Blaberoidea; Ectobiidae		PSN37932	1 protein isoform
Cryptotermes secundus		XP_023723334	2 protein isoforms

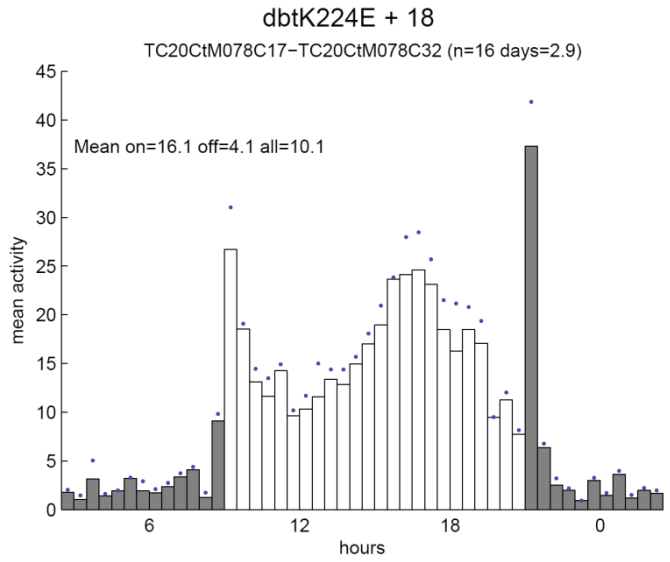
Insecta; Polyneoptera; Blattodea; Blattoidea; Termitoidae;		XP_023723336	
Ladona fulva Insecta; Pterygota; Palaeoptera; Odonata; Anisoptera;		KAG8233907	1 protein isoform
Ecdyonurus insignis Insecta; Pterygota; Palaeoptera; Ephemeroptera; Setisura;		GCCL01038339	1 protein isoform (identified in TSA)
Thermobia domestica Insecta; Zygentoma; Lepismatidae;		GASN02058628	1 protein isoform (identified in TSA)
Atelura formicaria Insecta; Zygentoma; Nicoletiidae;		GAYJ02042424	1 protein isoform (identified in TSA)
Strongylocentrotus purpuratus Echinodermata; Echinoidea; Camarodonta; Strongylocentrotidae		XP_779963	1 protein isoform
Acanthaster planci Echinodermata; Asteroidea; Valvatida; Acanthasteridae		XP_022080523 XP_022080521	2 protein isoforms
Saccoglossus kowalevskii Hemichordata; Enteropneusta; Harrimaniidae; Saccoglossus		XP_006813451 XP_006813452 XP_006813450 XP_006813449 XP_002732288 XP_006813448	6 protein isoforms
Branchiostoma floridae Chordata; Leptocardii; Branchiostomidae		XP_035696214	1 protein isoform
Petromyzon marinus Metazoa; Chordata; Vertebrata; Cyclostomata; Hyperoartia;	CKI LOC116955997	XP_032833279	1 protein isoform
	CKI-like LOC116949661	XP_032823110 XP_032823111	2 protein isoforms
Danio rerio Chordata; Vertebrata; Actinopteri; Teleostei; Cypriniformes;	CKI delta-a	NP_955877	1 protein isoform
	CKI delta-b	NP_998415	1 protein isoform
	CKI epsilon	NP_997912	1 protein isoform
Xenopus laevis Chordata; Vertebrata; Tetrapoda; Amphibia; Anura;	CKI delta-S	XP_018096015	1 protein isoform
	CKI delta-L	XP_018090607 XP_018090608 XP_018090609	3 protein isoforms
	CKI epsilon	XP_018112072	1 protein isoform
Anolis carolinensis Chordata; Vertebrata; Tetrapoda; Sauria; Lepidosauria; Squamata;	CKI delta	XP_003217335 XP_008102766 XP_008102767	3 protein isoforms
	CKI epsilon	XP_003227197	1 protein isoform
Gallus gallus Chordata; Vertebrata; Archosauria; Dinosauria; Aves; Neognathae;	CKI delta	XP_415634 XP_040505424	2 protein isoforms
	CKI epsilon	NP_989708	1 protein isoform
Vombatus ursinus Chordata; Vertebrata; Tetrapoda; Mammalia; Diprotodontia;	CKI delta	XP_027728943 XP_027728944 XP_027728945	3 protein isoforms
	CKI epsilon	XP_027727465 XP_027727484 XP_027727496	3 protein isoforms
Mus musculus Chordata; Vertebrata; Tetrapoda; Mammalia; Rodentia;	CKI delta	NP_620690 NP_082150	2 protein isoforms
	CKI epsilon	NP_001276828 NP_038795	2 protein isoforms

Homo sapiens Chordata; Vertebrata; Tetrapoda; Mammalia; Primates;	CKI delta	NP_001884 NP_620693 NP_001350678	3 protein isoforms
	CKI epsilon	NP_689407	1 protein isoform

Species/group	Gene	Protein acc number isoforms	note
Drosophila melanogaster Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha; Drosophilidae	1	AAN09313	CKIalpha
	1	AAL68089 / AT17410p	CKIalpha-like I, *Drosophila* genus unique
	1	NP_649536/CG12147	CKIalpha-like II, *Drosophila* genus unique
	1	NP_608697/CG9962	*Drosophila* genus unique CKI
	1	NP_732123/gilgamesh_isoA	gilgammesh/CKIgamma (splicing isoforms)
		AAF55294/gilgamesh_isoB	
		NP_524941/gilgamesh_isoC	
		AAN13704/gilgamesh_isoD	
		NP_788683/gilgamesh_isoE	
		NP_001014628/gilgamesh_isoF	
		NP_001014627/gilgamesh_isoG	
		NP_001163628/gilgamesh_isoI	
		NP_001247137/gilgamesh_isoJ	
		NP_001247138/gilgamesh_isoK	
		NP_001262624/gilgamesh_isoL	
		NP_001262625/gilgamesh_isoM	
		NP_001369018/gilgamesh_isoN	
		NP_001369019/gilgamesh_isoO	
		NP_001369020/gilgamesh_isoP	
	1	NP_572794/CG2577	*Drosophila* genus unique CKI
	1	NP_726577	asator / tau-tubulin kinase 1 (splicing isoforms)
		AAY55135	
Drosophila suzulii Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha; Drosophilidae	1	XP_016923997	CKIalpha
	1	XP_016928528	CKIalpha-like I, *Drosophila* genus unique
	1	XP_016931834	CKIalpha-like II, *Drosophila* genus unique
	1	XP_016932540	*Drosophila* genus unique CKI
	1	XP_036673801	gilgammesh/CKIgamma
	1	XP_016923903	*Drosophila* genus unique CKI
Drosophila willistoni Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha; Drosophilidae	1	XP_002070931	CKIalpha
	1	GJOF01005569	CKIalpha-like I, *Drosophila* genus unique
	1	EDW83428 1	CKIalpha-like II, *Drosophila* genus unique
	1	XP_002066575	*Drosophila* genus unique CKI
	1	XP_023035705	gilgammesh/CKIgamma
	1	GJOF01002639	*Drosophila* genus unique CKI
Drosophila virilis Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha; Drosophilidae	**1**	**XP_002058127**	CKIalpha
	1	XP_002058128	CKIalpha
	1	XP_002048901	CKIalpha-like I, *Drosophila* genus unique
	1	KRF79126	CKIalpha-like II, *Drosophila* genus unique
	1	XP_002052097	*Drosophila* genus unique CKI
	1	XP_032289459	gilgammesh/CKIgamma
	1	XP_002057057	*Drosophila* genus unique CKI
Drosophila grimshawi Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha; Drosophilidae	1	XP_001991332	CKIalpha
	1	XP_001991333	CKIalpha
	1	XP_001989259	CKIalpha-like I, *Drosophila* genus unique
	1	XP_001994022	CKIalpha-like II, *Drosophila* genus unique
	1	XP_001990762	gilgammesh/CKIgamma
	1	XP_001995815	*Drosophila* genus unique CKI
Ceratitis capitata Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha, Tephritidae	1	XP_004536733	CKIalpha
	1	XP_020718038	gilgammesh/CKIgamma splicing isoforms
		XP_020718035	
		XP_023158495	
		XP_023158494	
		XP_020718036	
		XP_023158492	
		XP_023158491	

		XP_023158493	
	1	XP_012160860	asator / tau-tubulin kinase
Musca domestica Insecta; Holometabola; Diptera; Brachycera; Cyclorrhapha, Muscidae	1	XP_005175423	CKIalpha
	1	XP_019894430	gilgammesh/CKIgamma splicing isoforms
		XP_019894431	
		XP_019894432	
		XP_019894433	
		XP_01989343	asator / tau-tubulin kinase
Anopheles albimatus Insecta; Holometabola; Diptera; Nematocera; Culicidae	1	XP_035784016	CKIalpha
	1	XP_035782093	gilgammesh/CKIgamma splicing isoforms
		XP_035782080	
		XP_035782088	
		XP_035782089	
		XP_035782092	
		XP_035782086	
		XP_035782091	
	1	XP_035784891	asator / tau-tubulin kinase
Culex quinquefasciatus Insecta; Holometabola; Diptera; Nematocera; Culicidae	1	EDS27991	CKIalpha splicing isoforms
		XP_038111213	
	1	XP_038116328	gilgammesh/CKIgamma splicing isoforms
		XP_038116330	
		XP_038116331	
	1	XP_038116441	asator / tau-tubulin kinase
Danaus plexippus Insecta; Holometabola; Lepidoptera; Ditrysia; Nymphalidae	1	XP_032523226	CKIalpha
	1	XP_032525620	asator / tau-tubulin kinase (splicing isoforms)
	1	OWR52845	
Bombyx mori Insecta; Holometabola; Lepidoptera;	1	NP_001037287	CKIalpha
Halyomorpha halys Insecta; Paraneoptera; Hemiptera; Heteroptera; Pentatomomorpha;	1	XP_014272268	CKIalpha
	1	XP_024217897	gilgammesh/CKIgamma splicing isoforms
		XP_024217016	
	1	XP_014270770	asator / tau-tubulin kinase (splicing isoforms)
		XP_014270778	
Thermobia domestica Insecta; Zygentoma; Lepismatidae;	1	GASN02046539	CKIalpha
	1	GASN02049146	gilgammesh/CKIgamma
	1	GASN02059192	asator / tau-tubulin kinase
Strongylocentrotus purpuratus Echinodermata; Echinoidea; Camarodonta; Strongylocentrotidae	1	XP_786391	CKIalpha splicing isoforms
		XP_030841481	
	1	XP_783545	CKIalpha-like
	1	XP_779900	CKIgamma
Branchiostoma floridae Chordata; Leptocardii; Branchiostomidae	1	XP_035672527	CKIalpha
	1	XP_035666971	CKIgamma
Petromyzon marinus Metazoa; Chordata; Vertebrata;	1	XP_032806746	CKIalpha splicing isoforms
		XP_032806745	
	2	XP_032821058	asator / tau-tubulin kinase
		XP_032819888	asator / tau-tubulin kinase

Cyclostomata; Hyperoartia;			
Mus musculus Chordata; Vertebrata; Tetrapoda; Mammalia; Rodentia;	1	EDL09767	CKIalpha splicing isoforms
		NP_001344429	
		NP_001344427	
		NP_001344428	
	3	NP_775277	CKIgamma-1
		NP_001153063	CKIgamma-2
		EDL09899	CKIgamma-3 splicing isoforms
		EDL09900	
		EDL28020	tau tubulin kinase 2
		BAB62004	Tau-tubulin kinase
		XP_006523508	tau-tubulin kinase 1 isoform X3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Mean on=16.1 off=4.1 all=10.1
mean activity
hours
0
5
10
15
20
25
30
35
40
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6
12
18
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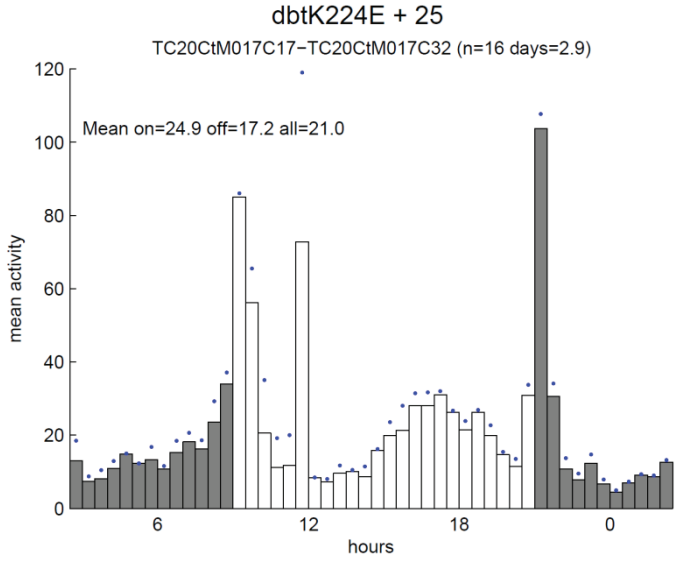
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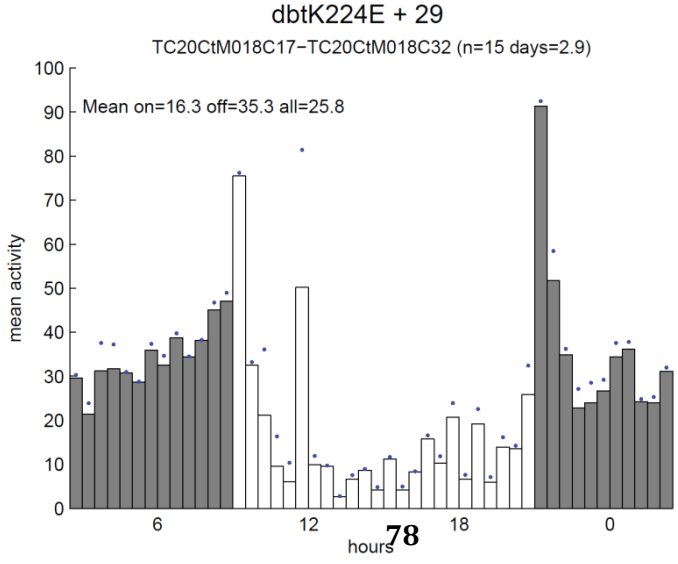
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hours
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60
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18
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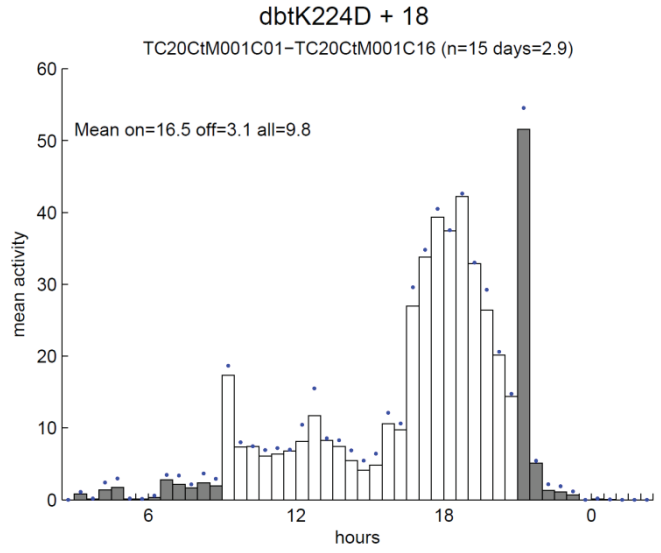
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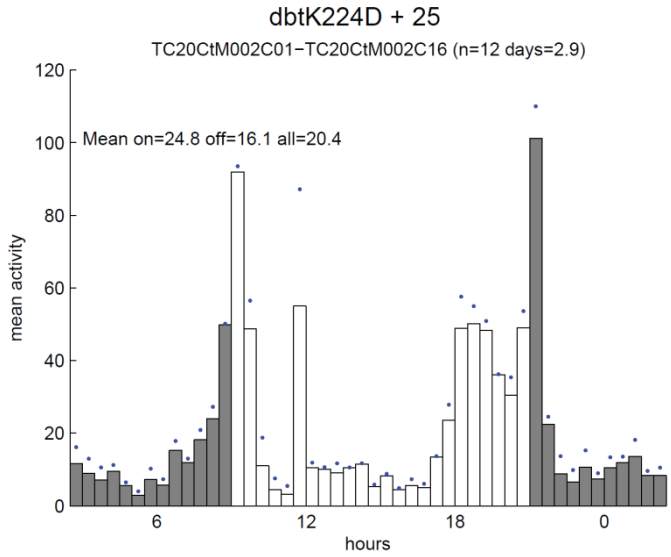
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hours
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60
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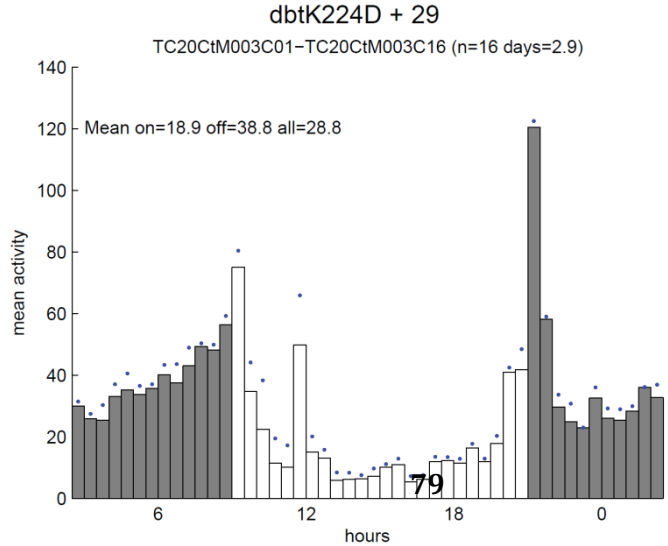
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6
12
18
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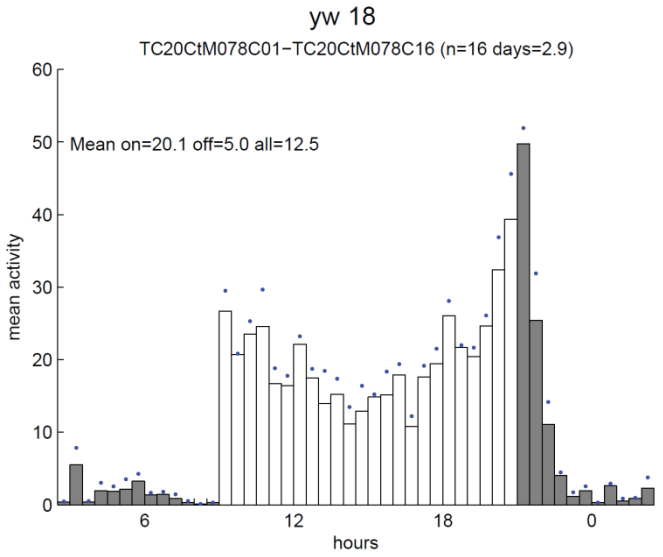
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mean activity
hours
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20
30
40
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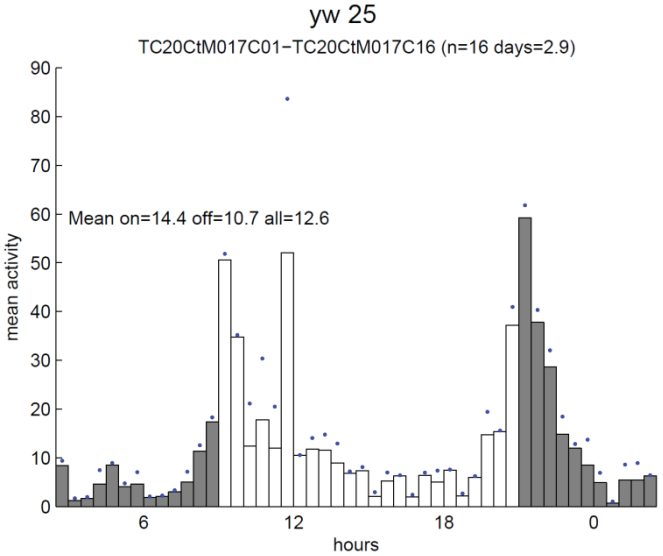
6
12
18
0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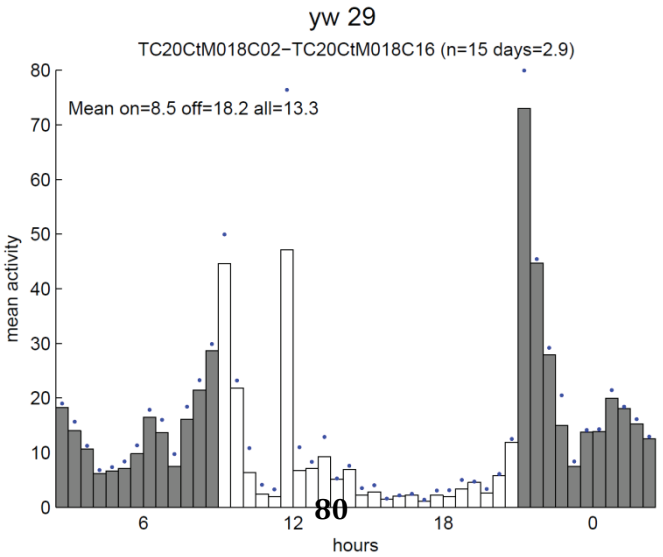
mean activity
hours
0
10
20
30
40
50
60
70
80
90
6
12
18
0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Mean on=8.5 off=18.2 all=13.3
mean activity
hours
0
10
20
30
40
50
60
70
80
6
12
18
0

Due to size and resolution limitation supplementary Files of Phylogenetics tree can be referred on the link given below

https://www.frontiersin.org/articles/10.3389/fphys.2022.1062632/full#supplementary-material

Part 2

Germline Editing of Drosophila Using CRISPR-Cas9-based Cytosine and Adenine Base Editors

Thakkar, N., Hejzlarova, A., Brabec, V., & Dolezel, D.

The CRISPR Journal, 2023

Since the development of CRISPR base editors in 2016, they were adapted and tested in some organisms, including crop plants such as rice, wheat, corn (Zong et al., 2017), animals such as zebrafish (Lu et al., 2018; Qin et al., 2018; Tanaka et al., 2018), mouse (Kim et al., 2017; Lim et al., 2020; Song et al., 2020) and the insect such as silkworm *Bombyx mori* (Li et al., 2018). Moreover, CRISPR base editor literature in *Drosophila* was quite scarce until recently, with one study testing CBE in somatic cells (surfaced in 2021) (Marr and Potter, 2021) and another study testing CBEs in germ cells (surfaced in August 2023) (Doll et al., 2023). Additionally, considering the limited number of precise point mutants of *Drosophila* circadian genes (given its length) and the requisite of understanding the protein motifs (as circadian gene functions have been meticulously established), we embarked on testing ABE and CBE in *Drosophila*, with the intention of their future applicability to editing circadian clock genes.

In this chapter, we have tested two CBEs (Target-AID and BE3) and one ABE (ABE7.10) for germline editing in the fruit fly *Drosophila melanogaster*. Moreover, we developed a construct (white-4gRNA) expressing multiple guide RNAs which can target splice sites in the X-chromosome-located white gene. By crossing fly lines expressing base editors (ABE and CBE) and white-4gRNA, we checked the efficiency of germline editing at three distinct temperatures. As a reference, we also used classical cas9 expressing line which rendered insertion/deletion mutations resulting from non-homologous end joining. Furthermore, we found out that base editing was most efficient at 28°C, the highest temperature that supports *Drosophila* survival. Ultimately, using the Target-AID cytidine base editor, we created a base-edited allele of the *timeless* gene ($tim^{SS308\text{-}9FL}$). Additionally, upon circadian locomotor activity analysis, we found that $tim^{SS308\text{-}9FL}$ mutants had a disrupted circadian clock with a free-running period of 29 hours.

The CRISPR Journal
Volume XX, Number X, 2023

DOI: 10.1089/crispr.2023.0026

The CRISPR Journal

ORIGINAL ARTICLE

Germline Editing of *Drosophila* Using CRISPR-Cas9-based Cytosine and Adenine Base Editors

Nirav Thakkar,[1,2,†] Adela Hejzlarova,[1,†] Vaclav Brabec,[1] and David Dolezel[1,2,*]

Abstract

Target-AID, BE3, and ABE7.10 base editors fused to the catalytically modified Cas9 and xCas9(3.7) were tested for germline editing of the fruit fly *Drosophila melanogaster*. We developed a guide RNA-expressing construct, *white*-4gRNA, targeting splice sites in the *white* gene, an X-chromosome located gene. Using *white*-4gRNA flies and transgenic lines expressing Target-AID, BE3, and ABE7.10 base editors, we tested the efficiency of stable germline gene editing at three different temperatures. Classical Cas9 generating insertions/deletions by non-homologous end joining served as a reference. Our data indicate that gene editing is most efficient at 28°C, the highest temperature suitable for fruit flies. Finally, we created a new allele of the core circadian clock gene *timeless* using Target-AID. This base edited mutant allele $tim^{SS308\text{-}9FL}$ had a disrupted circadian clock with a period of ~29 h. The *white*-4gRNA expressing fly can be used to test new generations of base editors for future applications in *Drosophila*.

Introduction

CRISPR-Cas9 revolutionized genetic engineering and became a widely used tool in reverse genetics. Essentially, a complex of Cas9 protein and guide RNA (gRNA) is targeted to a specific DNA sequence where a double-strand break (DSB) occurs. Sequence recognition requires complementarity of 20–23 nucleotides between the gRNA and the target DNA, as well as a specific three-nucleotide protospacer adjacent motif (PAM), which is directly recognized by Cas9.[1–4]

After a DSB is created, the cleaved DNA is repaired by a nonhomologous end-joining (NHEJ) or microhomology-mediated end-joining (MMEJ) repair mechanism, which subsequently leads to gene disruption through insertions-deletions (indels), translocations, and other DNA rearrangements.[5,6]

While the generation of null mutants, often generated after the introduction of DSB with CRISPR-Cas9, is a powerful approach to uncover gene function, more delicate and controllable DNA modifications are usually required to decipher protein functions. Therefore, several approaches have been developed to make precise and specific changes in DNA sequence instead of the unpredictable DNA rearrangements resulting from NHEJ and MMEJ.

For example, along with the introduction of DNA breaks, a DNA template is provided for the homology-directed repair. Alternatively, prime editors rely on information provided by prime editing guide RNA.[7]

A special group of genome-modifying enzymes are base editors, genetically engineered enzymes that can introduce point mutations without DSBs and without DNA template. Base editors consist of a DNA deaminase enzyme in combination with a catalytically impaired Cas9 nuclease that either generates only single-strand nicks (nCas9) or does not cleave DNA at all (dCas9). Further, some base editors contain additional enzyme domains, such as uracil glycosylase inhibitor (UGI), which help to maintain the mutation in the DNA.

Depending on the deaminase, two major groups of base editors are distinguished: cytosine base editors, which convert C/G pairs to T/A pairs, and adenine base editors (ABEs), which are responsible for the conversion of A/T to G/C pairs. The rat cytidine deaminase rAPOBEC1 was linked to dCas9 to produce a first-generation cytidine base editor (BE1),[8] which was further modified in several rounds to improve its efficiency. The third-generation cytidine base editor (BE3) had a mutation efficiency of nearly 75% and contained nickase-Cas9 (nCas9) and UGI as independent components.[8]

[1]*Biology Center of the Academy of Sciences of the Czech Republic, Institute of Entomology, Ceske Budejovice, Czech Republic and* [2]*Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic.*

[†]*Equal contributions.*

*Address correspondence to: *David Dolezel, Biology Center of the Academy of Sciences of the Czech Republic, Institute of Entomology, Branisovska 31, 37005, Ceske Budejovice, Czech Republic,* E-mail: dolezel@entu.cas.cz; david.dolezel@entu.cas.cz

Similarly, a cytidine deaminase from the sea lamprey PmCDA1 was harnessed with nCas9 in a system called Target-AID and achieved high efficiency of single nucleotide mutations.[9] In 2017, an ABE was produced by protein engineering of *Escherichia coli* adenine deaminase (TadA) that can convert A→G by deaminating adenine to inosine. Like BE3, ABE was also modified in several rounds to improve its efficiency and expand the targeting window in which ABE7.10 has optimal efficiency.[10]

However, genome targeting remained limited to the PAM, which is required for the specific Cas9. To overcome this challenge, *Streptococcus pyogenes* Cas9 (Sp Cas9) was engineered into xCas9 3.7 variant that has broader PAM compatibility with a binding sequence of NG, GAA, and GAT.[11]

While the earlier-described base editors are conceptually established, the particular activity has been tested so far in some organisms, including animals such as zebrafish,[12–15] mice,[16–19] plants such as rice, wheat, and corn,[20–23] and the insect silkworm *Bombyx mori*.[24]

This study was designed as a test of base editors for stable germline transformation in *Drosophila*. Since we targeted a clearly visible marker, the *white* gene, we were able to compare the efficiency of three base editors and classical Cas9. Further, we tested the efficiency at three biologically meaningful temperatures. We found a relatively high level of mosaicism, confirming the previous reports of maternal deposition of the gRNA or Cas9 into fruit fly eggs.[25–27] The fly line presented here, which expresses multiple gRNA constructs, may be used to assess the efficiency of new base editors in the future.

Finally, to prove the concept, we generated a new allele of *timeless* (*tim*), a well-established circadian clock gene, and characterized the new mutant. This mutant allele $tim^{SS308\text{-}9FL}$ exhibited disruption of the circadian rhythm with a period of ~29 h. In addition, a severe defect in temperature compensation was also observed when the $tim^{SS308\text{-}9FL}$ allele was present as a hemizygote.

During our attempt to test base editing in *Drosophila*, two studies surfaced where in one study, the authors verified base editing in somatic cells using dBE2 cytidine deaminase[28] whereas in another study, the authors explored somatic and germline base editing by engineered versions of the cytidine deaminase $dCBE^{evoCDA1}$ and $dCBE^{evoAPOBEC1}$.[29]

Material and Methods

Plasmids

To make *Drosophila* base editing constructs, we acquired four base editors from the Addgene plasmid repository: (1) xCas9(3.7)-BE3 construct that expresses the modified rat Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (rat APOBEC1); a cytidine deaminase (Addgene #108380) (hereafter referred as BE3) and (2) xCas9(3.7)-ABE(7.10) construct that expresses modified *E. coli* tRNA specific adenosine deaminase (*E. coli* tadA; Uniprot ID: P68398) (Addgene #108382) (hereafter referred as ABE7.10); both mammalian expression vectors originally made and later modified in David Liu's lab[11] with engineered Cas9 variant ''x-Cas9(3.7)'' broadening the PAM flexibility to GAA, GAT, and NG in addition to its canonical NGG. (3) pcDNA3.1_pCMV-nCas-PmCDA1-ugi PH1-gRNA(HPRT) construct, which expresses cytidine deaminase 1 from lamprey (PmCDA1) and gRNA (HPRT) in mammalian cells from Akihiko Kondo's lab[9] (Addgene #79620) (hereafter referred as Target-AID).

Base editor coding sequences were transferred to the pBFV-nosP-Cas9 construct from the Shu Kondo's lab[30] (Addgene #138402), which contains the *nanos* (*nos*) promoter and the ''attB'' sequence for *PhiC31-based* genome integration. See the Supplementary Material and Methods for details of cloning and Supplementary Figures S1 and S2 for schematic maps of plasmids.

Two multiple gRNA constructs derived from pCFD5 multiplex gRNA plasmid (Addgene #73914) containing the *Drosophila* U6.3 promoter, the ''attB'' sequence, and vermilion as phenotypic marker were used in this study. To construct the *white*-4gRNA plasmid, four gRNA sequences (Table 1) with CRISPR base editing window at the exon-intron boundaries were integrated into 43–73 bp primers flanking pCFD5 plasmid (see Table 2 for primers and Supplementary Fig. S3 for plasmid map).

A total of three fragments were prepared by PCR using the earlier-mentioned primers and the *Bbs*I-digested pCFD5 plasmid as template. These three fragments were cloned into the *Bbs*I-digested pCFD5 vector using the Gibson cloning kit (New England Biolabs) (for detailed protocol see Ref.[31]).

Table 1. List of guide RNA sequences

gRNA name	*Sequence*	*Target*
w gRNA 1	ACT*CACAT*TGTTCAGATGCT	exon1-intron1 of *white* gene
w gRNA 2	ATT*GCAGG*GTGACAGCGGAG	intron1-Exon2 of *white* gene
w gRNA 3	GCA*AACTG*AGTTTCAAAT	intron2-Exon3 of *white* gene
w gRNA 4	CTC*ACTAG*GAAAAGAAGTCGA	exon3-intron3 of *white* gene
tim gRNA 1	CGGAGAGCTCTGAGGATAAT	*tim* exon 4-ABE
tim gRNA 2	ACCTCGCCCCCAAACAGGG	*tim* exon 4-BE3
tim gRNA 3	TCCTCGGACAATGGCAGCAA	*tim* exon 4-Target-AID

Gray background indicates the predicted editing window; The splice sites are underlined and italicized. Residues predicted to be potentially edited in the *tim* gene are underlined and bold.

ABE, adenine base editor; gRNA, guide RNA.

Table 2. Primers for Gibson cloning

Primer	*Sequence*	*Used for*
PCR1fwd xABE1	GCGGCCCGGGTTCGATTCCCGGCCGATGCACGGAGAGCTCTGAGGATAATG TTTTAGAGCTAGAAATAGCAAG	*tim* 3gRNA cloning
PCR1rev xBE5rev	CCCTGTTTGGGGGGCGAGGTTGCACCAGCCGGGAATCGAACCC	*tim* 3gRNA cloning
PCR2fwd xBE5	ACCTCGCCCCCCAAACAGGGGTTTTAGAGCTAGAAATAGCAAG	*tim* 3gRNA cloning
PCR2rev xBE9rev	AGAGGTAGGATCAGAGGTCATGCACCAGCCGGGAATCGAACCC	*tim* 3gRNA cloning
PCR3fwd xBE9	TGACCTCTGATCCTACCTCTGTTTTAGAGCTAGAAATAGCAAG	*tim* 3gRNA cloning
PCR3rev AID3rev	ATTTTAACTTGCTATTTCTAGCTCTAAAACTTGCTGCCATTGTCCG AGGATGCACCAGCCGGGAATCGAACCC	*tim* 3gRNA cloning
Gibson White PCR1 fwd	GCGGCCCGGGTTCGATTCCCGGCCGATGCAACTCACATTGTTCAG ATGCTGTTTTAGAGCTAGAAATAGCAAG	White 4gRNA cloning
Gibson White PCR1 rev	CTCCGCTGTCACCCTGCAATTGCACCAGCCGGGAATCGAACCC	White 4gRNA cloning
Gibson White PCR2 fwd	ATTGCAGGGTGACAGCGGAGGTTTTAGAGCTAGAAATAGCAAG	White 4gRNA cloning
Gibson White PCR2 rev	ATTTGAAACTCAGTTTGCTGCACCAGCCGGGAATCGAACCC	White 4gRNA cloning
Gibson White PCR3 fwd	GCAAACTGAGTTTCAAATGTTTTAGAGCTAGAAATAGCAAG	White 4gRNA cloning
Gibson White PCR3 rev	ATTTTAACTTGCTATTTCTAGCTCTAAAACTCGACTTCTTTTCCTA GTGAGTGCACCAGCCGGGAATCGAACCC	White 4gRNA cloning

For targeting the *tim* gene, three gRNA sequences (Table 1) with CRISPR base editing window in exon 4 of the *tim* gene were cloned into the *Bbs*I-digested pCFD5 using identical strategy (PCR amplification, Gibson assembly) as for the *white*-4gRNA plasmid. See Table 2 for the primers and Supplementary Figure S4 for the map of *tim*-3gRNA plasmid.

Drosophila genetics

Plasmids were injected into *y[1] M{vas-int.Dm}ZH-2A w[*]; P{y[+t7.7]=CaryP}attP2* carrying docking site on the third chromosome (WellGenetics, Inc., Taiwan) and founder flies were balanced with TM6C, after which homozygous lines were established. For *white* gene editing, the crossing scheme depicted in Figure 1 was used. First, 10 single crosses were carried out for each base editor, in which virgin females homozygous for the base editor were crossed with males homozygous for the *white*-4gRNA construct.

From the progeny, ~20 virgin flies (2 from each vial) that were heterozygous for the base editor and *white*-4gRNA were collected and individually crossed with Canton-S males. In the next generation, males with mutant eyes were collected, snap-frozen, and stored at −80°C for later molecular verification. The same protocol was repeated in four biological replicates for each base editor at 18°C, 25°C, and 28°C.

In parallel, a comparable crossing scheme was performed with *nos*-Cas9, where virgin flies used were $y^2cho^2v^1$*; P{nos-Cas9, y+, v+}3A/TM6C, Sb Tb*. To test whether the low editing efficiency of base editors and *nos*-Cas9 might be caused by the *nanos* promoter, we performed an experiment utilizing *vas*-Cas9 transgenic line marked with 3xP3-GFP (Bloomington Drosophila Stock Center #51324, w[1118]; PBac{y[+mDint2]=vas-Cas9}VK00027).

The crossing scheme remained the same as mentioned earlier, except that 20 virgin flies heterozygous for the *vas*-Cas9 and white-4gRNA were collected (instead of 40) and individually crossed together.

For *tim* gene editing, three single crosses were set up at 25°C by using homozygous males with *tim*-3gRNA and homozygous virgin females with *nos*-Target-AID (both transgenes are located on chromosome III). Heterozygous *nos*-Target-AID/*tim*-3gRNA female progeny were crossed to If/CyO males.

From their progeny, males and virgin females with CyO balancer were collected (*tim* is located on the second chromosome, and individually crossed with If/CyO (~15 single crosses)). From the progeny of each individual cross, +/CyO males and virgins were collected to establish unique lines, each with identical second chromosomes balanced by CyO. The mutation was identified by sequencing the target region of *tim* in homozygous individuals.

Sequencing

For the *white* gene editing, we randomly selected ~15 single white-eyed males (5 from each temperature, if possible) from the individual cross for each base editor and canonical Cas9. DNA was isolated using the squish protocol, *white* gene regions PCR-amplified and sequenced as described earlier.[32] For *tim* gene editing, 24 males (each representing a different line) homozygous for the second chromosome were individually squished and the targeted region of *tim* was PCR-amplified, purified, and sequenced (primers in Supplementary Table S1).

Splice site prediction

Splice Site Prediction by Neural Network (https://www.fruitfly.org/seq_tools/splice.html) of Berkeley Drosophila Genome Project was used to predict splice sites in

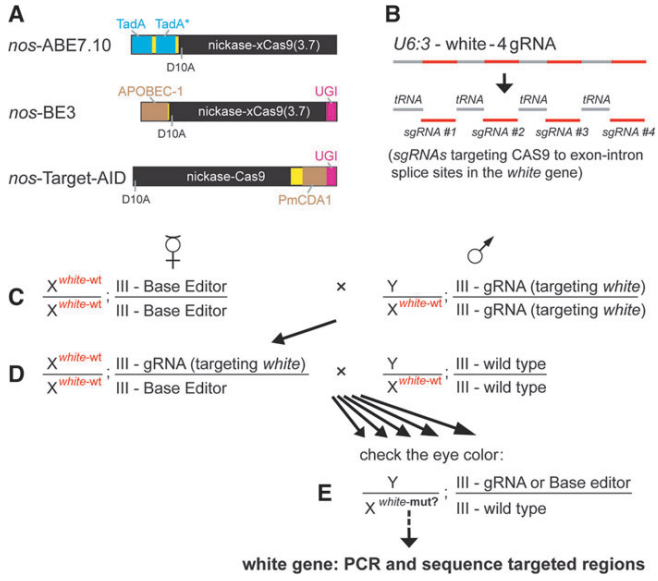


FIG. 1. Schematic description of here-tested constructs and a crossing scheme illustrating the targeting of the white gene in flies. **(A)** Major features of the three base editor proteins expressed from the *nos* promoters. **(B)** Four different gRNAs targeting the white gene at exon-intron splice sites are expressed from the U6:3 promoter as a single RNA transcript. Then, this transcript is processed by tRNA processing machinery and four sgRNAs are separated from the tRNA spacers. **(C)** Virgin females (left column) homozygous for the third chromosome containing the base editor (Target-AID, ABE7.10, or BE3) or CAS9 construct were crossed with males homozygous for a construct encoding multiple gRNAs targeting the white gene. Both males and females contain a wt allele of the white gene; thus, the eyes of these flies are red. **(D)** Virgins of the F1 progeny are heterozygous for both constructs and, therefore, produce base editors (or CAS9) and gRNA in the germ cells, which together can lead to editing of the white gene. The arrows in **B** indicate that multiple single female crosses were performed. **(E)** Successful editing is visually apparent in the sons of these females because the males are hemizygous for the white gene on the X chromosome. Mutagenesis is verified by Sanger sequencing of PCR products in a subset of males. gRNA, guide RNA; *nos*, *nanos*; sgRNA, single gRNA; tRNA, transfer RNA; wt, wild-type.

the *white* gene of selected mutants. The threshold score for 5′ and 3′ splice site prediction was set to 0.1 to predict any splice site even with the lowest score. However, any prediction score above 0.4 is considered optimal.

Genotyping mosaic males

To determine the genotype of males with mosaic eyes, individual flies were homogenized in 50 μL of squishing buffer. Transgene-specific primers were used in PCR, products electrophoretically separated, and the presence/absence of bands indicated whether the *white*-4gRNA construct or base editor/Cas9 construct was present. Control PCR was run with the original transgenic lines serving as a template to confirm the specificity of all primers.

Drosophila activity monitoring and analysis

CO_2-anesthetized, 3-day old males were housed in 5 mm glass tubes containing food (5% sucrose, 2% agar) and tubes were loaded into the Drosophila Activity Monitor system 2 (DAM2; Trikinetics, Waltham, USA). Flies were entrained for 5 days in a light:dark cycle of 12 h

(LD, 12:12), after which the lights were turned off and constant darkness (DD) was maintained for 12–15 days.

The activity was measured at 17°C, 20°C, 25°C, and 28°C. The first 10 days of DD were considered when calculating the free-running period (tau, τ) and the last 2–5 days served as a reference for the health/survival of the fly. From the activity data, double-plotted actograms were made using the ActogramJ plugin[33] and the Lomb-Scargle periodogram analysis was performed to find out the τ of the flies. Individual τ values and percentage rhythmicity data were plotted (GraphPad; Prism).

Results

Targeting the *white* gene

To test the efficiency of editing, disruption of the *Drosophila white* gene was used. This X chromosome-located gene facilitates efficient detection of mutation in males. Balancer chromosomes in *Drosophila* allow a reliable combination of gRNA and base editor transgenes, resulting in male mutant progeny after two genetic crosses (Fig. 1). Here, we explored cytidine (BE3, Target-AID) and adenine (ABE7.10) base editing in *Drosophila*, by driving their expression from germline-specific *nanos* promoter.

All three base editor constructs contain a nCas9, and BE3 and ABE7.10 constructs also contain engineered xCas9(3.7) variant permitting recognition to different PAM motifs such as NG, GAA and GAT[11] (Fig. 1A; Supplementary Fig. S2).

However, creating a null mutation by introducing a premature stop codon (TGA, TAA, or TAG) is possible only with cytidine base editors, whereas *de novo* production of any stop codon is not achievable with ABEs (stop codon can be obtained only from another stop codon). Therefore, to be able to use identical gRNAs for all tested base editors, we targeted the 5′ and 3′ splice recognition sites in the *white* gene with the aim of preventing the proper splicing of transcribed RNA to mRNA. To ensure a severe impact on the resulting protein, the exon boundaries of the first three exons were targeted (Fig. 3A).

The clearly visible eye color allowed us to phenotypically analyze around 20,000 male flies for each tested base editor, where *nos*-Cas9 served as a positive control. Further, we assessed the editing capacity at three temperatures. Flies with altered phenotype fell into two categories: complete eye color changed to white (sometimes slightly yellow), and flies with a mosaic distribution of white and red (wild-type) color. For evaluating the efficiency, we plotted the frequency of mutant flies identified per total number of flies (Fig. 2A, B, Supplementary Table S4) and the percentage of vials (that is offspring of a single cross) with at least one mutant fly (Fig. 2C, D, Supplementary Table S5 and Supplementary Fig. S5).

Comparison of the efficiencies with *vasa*-Cas9

To test whether the editing efficiency is influenced by the *white*-4gRNA construct, additional cross was performed with a highly active *vasa*-Cas9 line. As illustrated in Figure 2E and Supplementary Table S6, the data exclude the possibility that the low efficiency observed with the base editors and *nos*-Cas9 are due to inefficient gRNAs.

The efficiency is affected by the temperature

At an ambient temperature of 25°C, Target-AID was the most efficient base editor in generating white-eyed flies (0.4% of flies; 29% of vials/crosses), whereas the efficiency of BE3 and ABE7.10 was comparable (0.16–0.19% of flies; 13% of crosses). Reference *nos*-Cas9 generated approximately seven times more mutants than Target-AID (2.87% of flies; 70% of crosses; Fig. 2A, C; Supplementary Tables S2 and S3).

Because *Drosophila* does not control its body temperature, we compared the efficiency of base editors at 18°C, 25°C, and 28°C. At all temperatures, the trend of efficiency was similar (*nos*-Cas9 > Target-AID > BE3/ABE7.10). However, comparing the efficiency of the same editor at three temperatures showed different trends. *nos*-Cas9 was comparably efficient at all three temperatures (the differences were not statistically significant).

The efficiency of BE3 and ABE7.10 increased with temperature, whereas no editing was detected at 18°C and efficiency increased approximately fivefold from 25°C to 28°C. Target-AID was most efficient at 28°C, whereas efficiency was lowest at 25°C and intermediate at 18°C (Fig. 2A, C; Supplementary Tables S2 and S3).

Mosaicism

We identified a substantial number of males in which the compound eyes exhibited a distinctly mosaic distribution of pigment (Figs. 2B, D and 4B–E). In general, the trends were like those observed for white-eyed fly abundance. The highest frequency was observed for *nos*-Cas9 and then for Target-AID. While white-eyed flies were produced with comparable efficiency by BE3 and ABE7.10, mosaics were produced more frequently with ABE7.10 than with BE3. The frequency increased with temperature for each base editor and even for Target-AID (Fig. 2B, D).

Molecular changes introduced by base editors and *nos*-Cas9

White gene target regions were sequenced from ~15 males for each base editor and *nos*-Cas9. When possible, five males were selected for each temperature. Because the regions with four target sites had to be amplified in

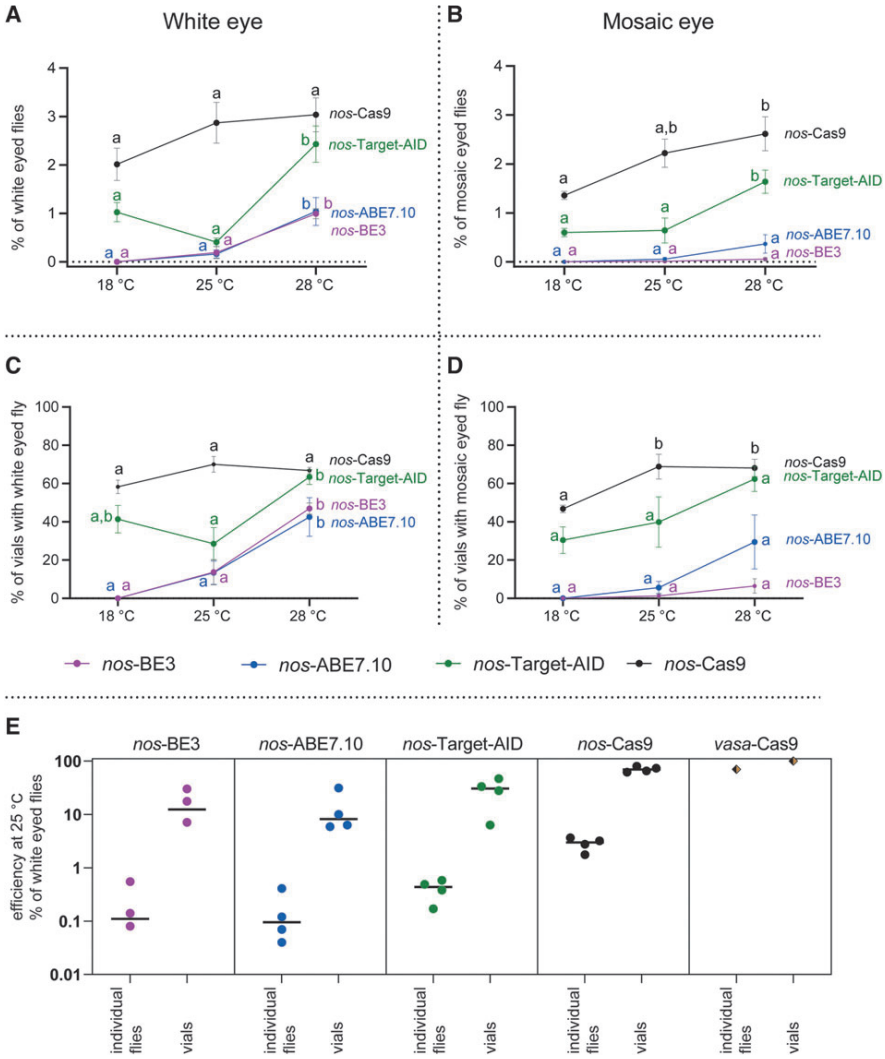
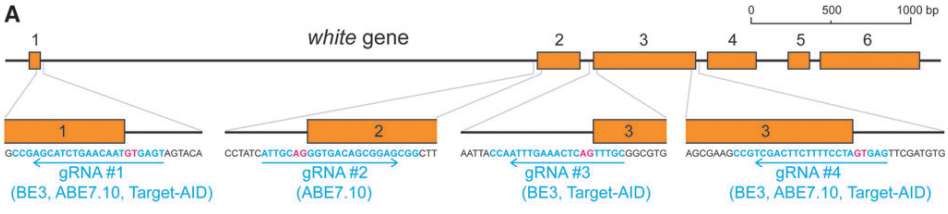


FIG. 2. Efficiency of white gene mutagenesis at three temperatures using the *Drosophila* germline expressed (*nanos* promoter) base editor and the canonical CRISPR Cas9 editor. **(A)** Percentage of individual flies with white eyes and **(B)** percentage of flies with mosaic eyes obtained from different genome editors at 18°C, 25°C, and 28°C are shown. **(C)** Percentage of vials with white-eye flies and **(D)** percentage of vials with mosaic-eye flies obtained from different genome editors at 18°C, 25°C and 28°C are shown. **(E)** Comparison of tested base editors and nos-Cas9 with highly active vasa-Cas9 line (only data from 25°C are compared; *x*-axis is in logarithmic scale). Colors represent different genome editors as shown on the side. The whiskers represent the mean ± SEM, and the small letters show the variance for each base editor with $p < 0.01$ significance. SEM, standard error of the mean.



B

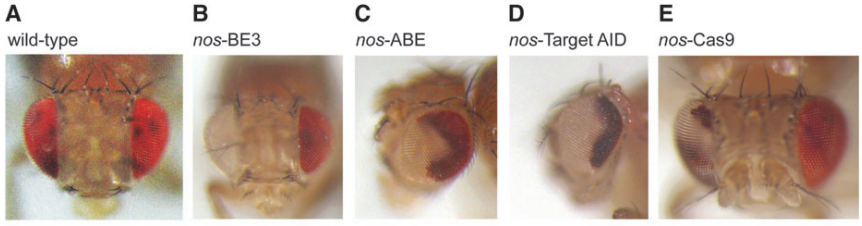
	gRNA #1				gRNA #2				gRNA #3				gRNA #4			
	edited	indels	intact	total	edited	indels	intact	total	edited	indels	intact	total	edited	indels	intact	total
nos-Target AID	15	3	4	(22)	0	2	20	(22)	0	0	22	(22)	2	1	19	(22)
nos-BE3	14	1	0	(15)	0	0	15	(15)	0	0	15	(15)	0	0	15	(15)
nos-ABE7.10	7	0	8	(15)	7	2	6	(15)	0	0	15	(15)	0	0	15	(15)
nos-CAS9	-	/11/	5	(16)	-	2	14	(16)	-	0	16	(16)	-	3	13	(16)

FIG. 3. Details of the white gene editing. **(A)** The gene consists of six exons. Four exon-intron boundaries were targeted by gRNAs #1–4, respectively. The gRNA sequence is depicted in blue, the orientation of the gRNA is indicated by the arrow, and the magenta color highlights the 5′ splice donor sites (exons 1 and 3) and the 3′ splice acceptor sites (exons 2 and 3). Theoretical prediction of which base editor should impact the splicing pattern is provided in parenthesis for each gRNA. **(B)** A summary of DNA modifications is depicted for each base editor at each region, with the number of individuals with edited DNA bases (edited), insertion and deletion (indel), and unmodified sequence (intact). The complete sequences are available in Supplementary Figures S6–S10.

three separate PCR reactions, we assembled the outcomes into one merged sequence that allows to identify and interpret co-occurrence of modifications (Supplementary Figs. S6–S9). A summary of the sequencing is depicted in Figure 3B.

The efficiency varied between target sites: The region targeted by gRNA1 was edited most frequently, whereas no editing was detected for region targeted by gRNA3. Further, a few deletions were also observed.

Sequence analysis revealed that the 5′ splice donor site was precisely modified by Target-AID at #1 and #4 (Supplementary Fig. S6) whereas BE3 modified it only at #1 (Supplementary Fig. S7; note that the reverse DNA strand was targeted in #1 and #4, therefore C-to-T



G

	nos-Target AID	*nos*-ABE	*nos*-BE3	*nos*-Cas9
gRNA transgene	56 (60%)	16 (67%)	2 (40%)	45 (49%)
Base editor transg.	37 (40%)	8 (33%)	3 (60%)	47 (51%)

FIG. 4. Eye color mosaicism was detected in a subset of F2 males. **(A)** Complete pigmentation in wt male (control). **(B)** BE3-induced mosaic with a red left eye and a white right eye. **(C)** ABE-induced and **(D)** Target-AID-induced mosaicism in the portion of the left eye. **(E)** CAS9-induced mosaicism. **(F)** Schematic depiction of the male F2 genotype was clarified by transgene-specific PCR. **(G)** Table summarizing the frequency of the transgene (base-editor or gRNA) present in F2 mosaic males.

modifications are depicted as G-to-A in the Supplementary Figs. S6 and S7). Region #1 illustrates the difference in editing specificity: within a five-nucleotide window, Target-AID modified three cytidines (six flies), two cytidines (three flies), or only one cytidine (four flies).

In one fly, a cytidine located seven nucleotides upstream of the window was also modified. BE3 modified either two cytidines (13 flies) or one cytidine (1 fly). Moreover, two C-to-G changes in the editing window were also modified by Target-AID (Supplementary Fig. S6).

ABE7.10 precisely modified ''A'' to ''G'' in the 5′ splice donor site at #1 (reverse strand was targeted) and in the 3′ splice acceptor site at #2 (Supplementary Fig. S8). Contrary to cytidine base editors, in the case of ABE7.10, additional ''A'' at #1 was not modified despite it falling in the editing window (Supplementary Fig. S8).

Canonical CRISPR-Cas9 editing destroyed the target region due to several indels in #1, #2, and #4 (Supplementary Fig. S9; Fig. 3B). Contrarily, the Target-AID, BE3, and ABE7.10 scarcely disrupted the target DNA in regions #1, #2, and #4, resulting in very few indels (Supplementary Figs. S6–S8; Fig. 3B). Notably, the indels generated by the base editors differed from those of the *nos*-Cas9, comprising mainly deletions, whereas the Cas9-created indels carried insertions and deletions (Supplementary Figs. S6–S9).

A small subset of the flies with completely changed eye color was slightly yellowish. We sequenced seven such flies generated with Target-AID and one with *nos*-Cas9 (labelled ''yellow'' in Supplementary Figs. S6 and S9). Three of them contained short deletion, whereas five of them (all from Target-AID) contained only one nucleotide substitution downstream of the splice site resulting in a prediction of less efficient splice recognition site by Splice Site Prediction Tool Neural Network (0.27 or 0.66 instead of the 0.88 in the original sequence; Supplementary Fig. S10).

A subset of F2 males exhibited a distinct mosaic eye. When the *white* gene was PCR amplified from selected mosaic males and sequenced, we could see clean chromatograms that abruptly changed to a chromatogram in which multiple peaks overlapped (Supplementary Fig. S11). The position where this change occurs corresponds exactly to the predicted editing site. Since some flies contained deletions even after base editors were applied, we assume that a subset of the *white* genes contains a deletion that leads to the observed sequencing pattern.

Genotype of mosaic males

Males of F2 generation (Fig. 1E) should contain either a gRNA-encoding construct or a construct with base editor, but never both (all constructs landed at the identical docking site on the third chromosome, so that even recombination in the females [Fig. 1D], could not lead to the presence of both constructs in F2 males).

To clarify the genotype of the F2 males, PCR was used to detect the presence of gRNA and base editor constructs. First, we indeed confirmed the presence of just one construct in each F2 male. In all three base editor and in *nos*-Cas9 crosses, either the gRNA construct or the base editor construct (Cas9) was detected. Enough flies with mosaic eyes were available only for Target-AID and *nos*-Cas9; their analysis indicates approximately equal presence of gRNA or base editor (Cas9) constructs (Fig. 4G). Accordingly, either the gRNA or the base editor protein (or its mRNA) must be deposited maternally into the egg.

Targeting circadian clock gene *tim*

Given the successful modification of a gene with a clearly visible phenotypic change, we tested the applicability of the gene editing protocol for the introduction of mutations that might be informative for a specific research question. The target was *tim*, a gene that is a well-established component of the circadian clock,[34–36] but because of its length, several regions have not previously been targeted experimentally.

First, we performed *in silico* analysis of the *Drosophila* TIM protein across various insect species and identified a highly conserved region similar even in the most basal insect *Thermobia domestica* (Fig. 5A). Moreover, a few point mutants with divergent circadian phenotypes have been described in this region, but the regulatory mechanism is not completely understood[32,37,38] (Fig. 5A).

Therefore, we targeted two highly conserved serines (S) at positions 308–309 in TIM (numbered according to the L-TIM isoform; 1421 aa) with the DNA coding sequence of TCC and TCG (Fig. 5A; Supplementary Fig. S12) using the Target-AID. Finally, from the mutagenic screen, we sequenced 24 lines and identified a base-edited *tim* mutant (~4% editing efficiency) with the modified DNA coding sequence of TTT and TTG encoding for phenylalanine (F) and leucine (L) ($tim^{SS308\text{-}9FL}$) (Fig. 5A top; Supplementary Fig. S12).

To determine the impact of the mutation on the *Drosophila* circadian clock, we analyzed the locomotor activity of the $tim^{SS308\text{-}9FL}$ mutant at four different temperatures (17°C, 20°C, 25°C, and 28°C). As shown in Figure 5B, the free-running period (tau, τ) of wild-type flies (canton-s) is nearly 24 h ($\tau \backsim 23.5$ h) over the entire physiological temperature range of *Drosophila*.

In agreement with previous studies,[32,39] even the single copy of wild-type *tim* is sufficient to keep the clock ticking at the same pace at 17°C, 20°C, 25°C, and 28°C (tim^{01}/+; $\tau \sim 23.5$ h) (Fig. 5C, G; Supplementary

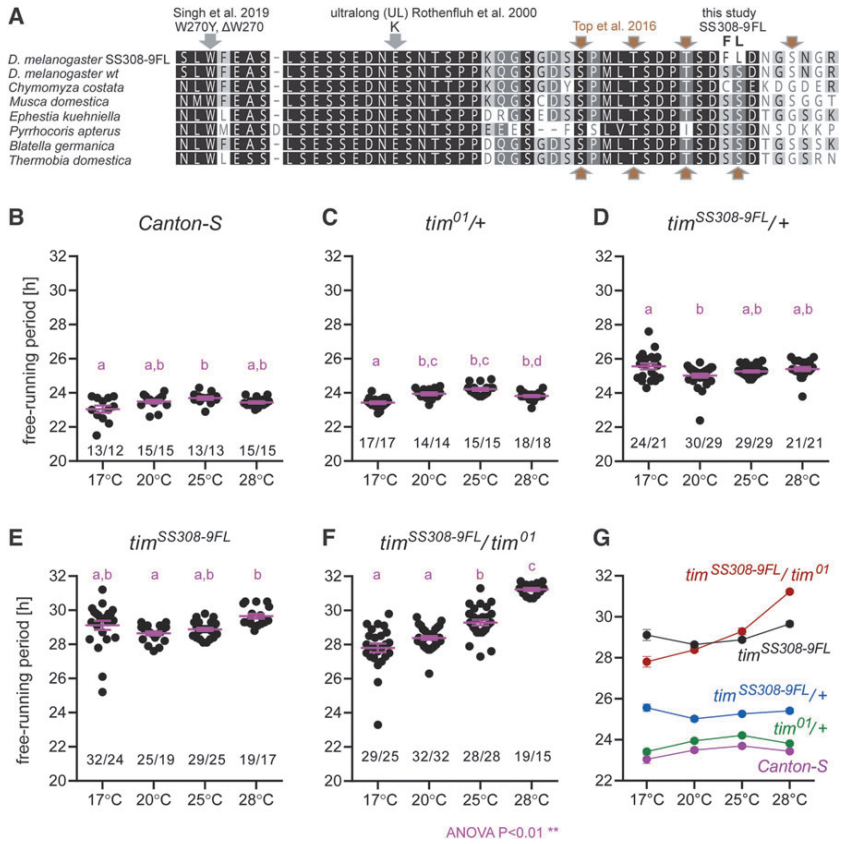


FIG. 5. Functional analysis of the Target-AID-edited *timeless* mutant. **(A)** Alignment of the highly conserved region of TIM protein among representative species from different insect groups and the *Drosophila melanogaster* mutant TIM generated here with a change of two amino acids SS308-309 to FL ($tim^{SS308\text{-}9FL}$), shown as the top sequence in the alignment. Known mutations in this region are depicted above the alignment. **(B–F)** Circadian phenotype was recorded for 8–10 days in DD for the given temperatures, and each black dot in panels **(B–F)** represents the free-running period (tau, τ) of individual male flies. Numbers at the top of *x*-axis represent $n_{total}/n_{rhythmic}$. Magenta bars represent the mean ± SEM, and magenta small alphabet letters represent the variance with $p < 0.01$ significance. **(G)** Comparative analysis of the free-running period of the different genotypes at specific temperatures. TIM, TIMELESS.

Fig. S13). Surprisingly, the base-edited *timeless* mutant $tim^{SS308\text{–}9FL}$ allele was found to be dominant over wild-type *tim* and slowed down the circadian clock by nearly 1.5 h ($tim^{SS308\text{-}9FL}$/+ heterozygote; $\tau \backsim 25$ h; Fig. 5D, G; Supplementary Fig. S13).

In $tim^{SS308\text{–}9FL}$ homozygotes, the circadian clock slowed by as much as 5.5 h ($\tau \backsim 29$ h) compared with wild-type flies (Fig. 5E; Supplementary Fig. S13). Further, $tim^{SS308\text{-}9FL}$ hemizygotes showed impaired temperature compensation, with the clock running faster at 17°C

($\tau \backsim 28$ h) and slower at 28°C ($\tau \backsim 31$ h) (Fig. 5F, G; Supplementary Fig. S13).

Discussion

The fact that base editors do not generate DSB makes them a promising tool for gene therapy. Originally, base editors were developed in mammalian cell culture assays with the intention of rectifying single nucleotide errors in human genetic diseases. Nonetheless, given their potential as a scarless gene editing tool, CRISPR base editors were adapted in several model organisms such as mice,[16–19] zebrafish,[12–15] the silkworm *B. mori*,[24] and various plant species.[20–23]

However, the base editors remained to be tested in *Drosophila melanogaster*, a key model indispensable for insect physiology and human translational research.[40,41] Therefore, we introduced two cytidine (Target-AID, BE3) and one adenine (ABE7.10) base editors in the *Drosophila* system.

During our endeavor, one study addressed dBE2 (second-generation cytidine base editor) in *Drosophila* somatic cells,[28] whereas another study explored somatic and germline base editing of the cytidine deaminases $dCBE^{evoCDA1}$ and $dCBE^{evoAPOBEC1}$.[29]

Notably, even the highest efficiencies of our base editors were very low (0.9–2.4% at 28°C; Supplementary Table S2) contrasting with 70–95% of $dCBE^{evoCDA1}$ and $dCBE^{evoAPOBEC1}$.[29]

Since epigenetic features or local molecular environment affects targeting by Cas9 regardless of prediction score,[42–44] we tested the efficiency of *white*-4gRNA to rule out that the construct produces inefficient gRNAs (Fig. 2E). The editing efficiency is further impacted by expression of Cas9/base editor, that is, the combination of a particular promoter in the construct and the landing site.[45,46] Indeed, some *nos*-Cas9 lines (i.e., CAS0003) have low activity whereas the same construct inserted elsewhere in the genome is more efficient.[46]

Since here used attP2 landing site supports good transgene expression, the low efficiency might be connected to the base editor design. Possible explanations include Cas9 nuclease modifications to either curb indels or broaden the PAM recognition sequence. Although xCas9(3.7) nickase-based editors are less effective than the canonical Cas9-derived target-AID, additional changes in these base editors outside of Cas9 could contribute, such as N- or C-terminal orientation of the deaminase affecting the editing window, spacers affecting the structural flexibility of the deaminases, and the dosage of nuclear localization signal sequence.

Depending on the intent of the experiment (efficiency vs. lethality), CRISPR base-editors expressed from different drivers are required. Target-AID, BE3, and ABE7.10 expressed from *nanos* promoter serve the purpose of germline restricted base editing.

In our study, we compared the efficiency of base editors expressed in flies at three temperatures (18°C, 25°C, and 28°C) from the *nanos* promoter (Supplementary Fig. S3) and the tRNAs flanking multiple gRNAs (targeting distinct exon-intron regions of the *white* gene) from the RNA Pol III promoter U6 (Supplementary Fig. S1). The CRISPR base editors were functional at 25°C (Fig. 2A; Supplementary Fig. S4A).

However, more male flies with white eyes were found at 28°C by CRISPR base editor mutagenesis, where editing by Target-AID was nearly threefold more efficient than BE3 and ABE7.10 (Fig. 2A; Supplementary Fig. S4A). Similarly, with increasing temperature, Doll et al. also observed higher C to T editing efficiency, where editing by $dCBE^{evoCDA1}$ was nearly two folds more efficient than $dCBE^{evoAPOBEC1}$ at 28°C.[29]

A similar temperature trend was also described with canonical CRISPR-Cas9 mutagenesis where the mutation efficiency increased several fold when *Arabidopsis* plants were subjected to heat treatment at 37°C.[47,48] Moreover, the effect of hyperthermia on CRISPR Cas9-mediated genome editing was observed in mammalian cell lines with most DNA modifications at 39°C.[49] Interestingly, we did not find a similar trend in our canonical CRISPR Cas9 mutagenesis in *Drosophila* (Fig. 2A; Supplementary Fig. S4A).

Further, an increase in knock-in and knock-out efficiency of the CRISPR-Cas9 tool was observed when the African clawed frog *Xenopus laevis* embryos were incubated for a specified time at a lower temperature after microinjection.[50] However, at 18°C, the efficiency of canonical CRISPR-Cas9 decreased in *Drosophila* (Fig. 2A; Supplementary Fig. S4A).

Strangely, the efficiency of Target-AID increased at 18°C compared with 25°C, making it the candidate base editor that can be used at low temperatures (Fig. 2A; Supplementary Fig. S4A). $dCBE^{evoCDA1}$ (engineered version of the lamprey cytidine deaminase) used in the concurrent study on *Drosophila* was also functional at 18°C, however its efficiency decreased compared with 24°C.[29] Perhaps, the functioning of the Target-AID at a lower temperature can be attributed to the higher activity of lamprey cytidine deaminase at a lower temperature (the host organism, sea lamprey *Petromyzon marinus*, lives in cold water).[51]

Notably, we obtained a few deletion mutants from Target-AID, BE3, and ABE7.10 (Fig. 3B; Supplementary Figs. S6–S8). Likewise, a small number of indels were also reported in the original studies in which these base

editors were developed (typically ≤1% for BE3, <0.3% for Target-AID, and ≤0.1% for ABE7.10),[8–10] in the silkworm *B. mori* (≤0.6% for BE3)[24] and in the study describing base editing in *Drosophila* (≤0.5% for dCBE^{evoAPOBEC1} and ≤2.3% for dCBE^{evoCDA1}).[29]

Such indels result from rare circumstances when the base excision repair pathway gets activated first, leading to the removal of the deaminated base and creation of a nick. Consequently, this causes DSB that leads to insertion and deletion-prone NHEJ.[52,53] Over the coming years, cytidine base editors have been improved to decrease the indels.[54–56] Thus, possible base editors with more precise editing capacity are available. The versions of base editors selected here combine some level of mutagenic capacity with precise editing so that they can be used to generate a wide range of mutants in conserved and less explored genomic regions.

While the original purpose of base editors requires editing to be as precise and focused as possible, base editors also open the possibility of becoming a tool for targeted mutagenesis. In this case, a restricted but broader window of up to tens or even hundreds of nucleotides would provide remarkable benefits. Although some base editors of this type have already been developed,[57] this line of research is still relatively underexplored. Another strategy that leads to a broader editing window is the simultaneous use of multiple gRNA.

Originally, multiple gRNA constructs in combination with Cas9 provided highly efficient gene deletions, even in a tissue-specific manner.[31] Combining base editors with multiple gRNA, several different targets can be edited with a possibility of different combinations of edited variants. In addition, editing diversity can be increased by using different base editors with the same set of gRNAs.

As we show here, the same gRNA leads to distinct editing patterns depending on the base editor; in the case of Target-AID, even various degrees of editing were observed at the same locus. Notably, simultaneous use of two gRNA spaced ~50 bp apart broadened the region modified by cytidine base editors.[28]

Like the F2 generation mosaic-eyed flies encountered in this study (Figs. 1E and 4F), various studies have reported non-mendelian trespassing of CRISPR-Cas9 components during oocyte maturation[25–27] which should be considered when doing gene manipulations using CRISPR-Cas9 and base editors.

As an example of a successful application of base editing, we created a new mutant of the *tim* gene. In principle, we used a similar approach as before,[32] except that Cas9 was replaced by Target-AID. The obtained mutation extended the free-running period similarly to the 3A mutant combining three substitutions T305A/S309A/S313A that prevent phosphorylation.[38]

The 3A mutant had 27-h-long behavioral rhythms, whereas in *tim*$^{SS308–9FL}$ homozygotes, the circadian clock was slowed to ~29 h at 25°C. Interestingly, the obtained mutant showed a dose-dependent defect in temperature compensation. A similar trend was observed for several other (but not all) *tim* mutants.[32,58] However, temperature compensation is not limited to the *tim* gene.

Temperature-dependent changes in the free-running period have been observed in the *period* gene[59–61] and in K224D mutants of *doubletime*,[62] a casein kinase I homolog responsible for PERIOD phosphorylation. Therefore, mutagenesis of clock proteins should be performed with the goal of identifying novel alleles responsible for temperature compensation. *Drosophila tim* gene studied here appears to function differently in some non-model insects as revealed from loss of function mutants.[63,64] Thus, precise editing tools that go beyond loss-of-function are needed in these species to decipher underlying differences. Base editors might be a suitable tool for such tasks.

Conclusions

Base editing expands the already broad toolkit of reverse genetics in *Drosophila*. In addition to being able to make precise gene changes in narrow windows, base editors can also serve as mutagenic tools, in which case a wider window or simultaneous expression of multiple gRNAs is preferable. The three base editors tested here demonstrate that distinct modifications can be achieved with identical gRNAs. The white multiple-gRNA fly (*white*-4gRNA) can serve as a reporter line to measure the efficiency of new base editors in the future. Finally, the efficiency of base editing in *Drosophila* is temperature dependent.

Acknowledgments

The authors thank Vlastimil Smykal for photography assistance, Shu Kondo for *nos*-Cas9 expressing flies and pBFV-nosP-Cas9 (Addgene #138402), Tomas Dolezal for *vas*-Cas9 expressing flies (BDSC #51324), Port and Bullock for pCFD5 multiplex gRNA plasmid (Addgene #73914), David Liu and Akihiko Kondo for plasmids encoding base-editors (Addgene #108380, 108382, and 79620), and two anonymous reviewers for constructive comments.

Authors' Contributions

Conceptualization of the study was done by D.D.; formal analysis was performed by N.T., A.H., and V.B.; investigation was done by N.T., A.H., and V.B.; original draft of

the article was written by N.T.; writing, review, and editing of the article was done by N.T and D.D.; funding acquisition was done by D.D.; supervision of the study was done by D.D. All authors approved the final article.

Disclaimer

The article has been submitted solely to this journal and is not published, in press, or submitted elsewhere.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This study was entirely supported by the Czech Science Foundation (GACR) Grant Number 22-10088S to D.D.

Supplementary Material

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Received: April 21, 2023
Accepted: October 2, 2023
Online Publication Date: November 2, 2023

Supplementary Material and Methods

Drosophila Base Editing Constructs – extended description

To make *Drosophila* base editing constructs, we acquired four base editors from the addgene plasmid repository; **A)** xCas9(3.7)-BE3 construct that expresses the modified rat Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (rat APOBEC1); a cytidine deaminase (Addgene# 108380) [hereafter referred as BE3] and **B)** xCas9(3.7)-ABE(7.10) construct that expresses modified *E.coli* tRNA specific adenosine deaminase (*E.coli* tadA) (Addgene#108382) [hereafter referred as ABE7.10]; both mammalian expression vectors from David Liu lab[1] **C)** pcDNA3.1_pCMV-nCas-PmCDA1-ugi PH1-gRNA(HPRT) construct which expresses Cytidine Deaminase 1 from lamprey (PmCDA1) and gRNA (HPRT) in mammalian cells from Akihiko Kondo lab (Addgene# 79620) [hereafter referred as Target-AID].[2] As these constructs were designed to express in mammalian systems, it was indispensable to change the vector for *Drosophila* genome integration as well as germline expression. For this reason, we used the pBFV-nosP-Cas9 construct from the Shu Kondo lab[3] (Addgene# 138402), which contains the *nanos* (*nos*) promoter and the 'attB' sequence for *PhiC31-based* genome integration.

From the pBFV-nosP-Cas9 construct, the 4,184 bp DNA sequence expressing canonical Sp Cas9 was excised using XbaI/AgeI restriction enzymes. A short linker containing XbaI/AgeI overhang with 37 bp (Linker 1) (See Fig S1) possessing multiple cloning sites was used to circularize the pBFV-nosP construct making "pBFV-nosP + Linker 1".

Drosophila nos-BE3 and *nos*-ABE construct

The NotI/PmeI digested 5,201 bp fragment from the BE3 construct (Addgene# 108380) containing rat APOBEC1 cytidine deaminase, nickase Cas9 (nCas9), Uracil Glycosylase Inhibitor (UGI), and the 5,429 bp fragment from the ABE7.10 construct (Addgene#108382) containing *E.coli* adenosine deaminase and nCAS9 were ligated to the NotI/PmeI digested 7,394 bp fragment of the pBFV-nosP + Linker 1 construct to make the *Drosophila nos*-BE3 construct (12,591 bp) and *Drosophila nos*-ABE7.10 construct (12,819 bp).

Drosophila nos-Target-AID construct

The XbaI/NheI digested 5,440 bp fragment from the Target-AID construct (Addgene# 79620) containing nCas9, lamprey cytidine deaminase and UGI was ligated to the XbaI/NheI digested 7,394 bp fragment of the pBFV-nosP + Linker 1 construct to make the *Drosophila nos*-Target-AID construct (12,826 bp).

All positive clones of the *Drosophila* base editing constructs were sequence verified using BE Clon F1 and BE Clon R2 primers (see Table S1).

Supplementary References:

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Supplementary Table 1: List of Primers used for sequence verification and diagnostic PCR

Primer	Sequence	Used for
BE Clon F1	CGTACGCTTCGCAGTTGTTT	BE Clone verification
BE Clon R2	CGAAATGAAGGCGACCAGTTG	BE Clone verification
U63seqfwd	ACGTTTTATAACTTATGCCCCTA	gRNA Clone verification
pCFD5seqrev	GCACAATTGTCTAGAATGCATAC	gRNA Clone verification
APOB1_Fw	ATGCGGCGAATGTAGTAGGG	*nos*-BE3 verification
APOB1_Rv	TGTGGCTGCTTCCTTCTCAG	*nos*-BE3 verification
CDA1_Fw	GCTGCTACGTGCTGTTTGAG	*nos*-Target-AID verification, mosaicism
CDA1_Rv	TGGTTCCGGGCATTCTTCTC	*nos*-Target-AID verification, mosaicism
TadAFw	CAGGAGATCAAGGCCCAGAA	*nos*-ABE7.10 verification, mosaicism
TadA*Rv	GTGGAGTCCGATTGCCCTAT	*nos*-ABE7.10 verification, mosaicism
nos Cas9 Fw1	ATCGAACCCTTGCCACCATG	*nos*-Cas9 verification, mosaicism
nos Cas9 Rv1	GTCTCCCCGGAGTCGAACAG	*nos*-Cas9 verification, mosaicism
WT1 Fw	TGCTGTGCCAAAACTCCTCT	White target site 1 detection
WT1 Rv	AATGAGGACTTCATGGTAAGCT	White target site 1 detection
WT2-3Fw	GGCTGGGCTAGATTTATGCAC	White target site 2-3 detection
WT2-3Rv	AAGCTCCTGGATCACCTGAT	White target site 2-3 detection
WT4-fw2	CTCCGAGGCTCTAACCGATC	White target site 4 detection
WT4-Rv2	TTTTGGTGGCCAACAACTGC	White target site 4 detection
ULBEdet_Fw	GCACATTCCAGAGACGCATG	tim editing detection
ULBEdet_Rv	GTTCCCCGTCATATCGCTGG	tim editing detection
w gRNA fw1	TCAGTGGTAGAATGCTCGCC	*white*-mgRNA verification - mosaicism
w gRNA R1	TAAAACCTCCGCTGTCACCC	*white*-mgRNA verification - mosaicism
APOB1_Fw2	TCGAGCCCCATGAGTTTGAG	*nos*-BE3 verification - mosaicism
APOB1_Rv2	GTGGTACAGCCTTGCGATGT	*nos*-BE3 verification - mosaicism

Supplementary Table 2

% of flies with white eyes					
			***nos*-BE3**		*average*
18 °C	0	0	0	0	0
25 °C	0.14	0.55	0.08	0	0.1925
28 °C	1	0.7	1.14	1.13	0.9925
			***nos*-ABE7.10**		
18 °C	0	0	0	0	0
25 °C	0.12	0.04	0.07	0.41	0.16
28 °C	1.28	0.44	1.73	0.71	1.04
			***nos*-Target-AID**		
18 °C	0.75	0.72	1.56	1.07	1.025
25 °C	0.17	0.49	0.58	0.38	0.405
28 °C	3.36	2.28	1.54	2.54	2.43
			***nos*-Cas9**		
18 °C	1.85	3	1.6	1.6	2.0125
25 °C	1.76	3.74	2.76	3.22	2.87
28 °C	3.67	2.63	2.26	3.58	3.035

Supplementary Table 3

% of vials (= crosses) with white editing events					
			***nos*-BE3**		*average*
18 °C	0	0	0	0	0
25 °C	17.6	30	7.1	0	13.675
28 °C	44.4	40	53.3	50	46.925
			***nos*-ABE7.10**		
18 °C	0	0	0	0	0
25 °C	10	5.9	6.3	31.3	13.375
28 °C	41.2	26.3	71.4	31.3	42.55
			***nos*-Target-AID**		
18 °C	54.2	20.8	46.7	43.8	41.375
25 °C	6.3	27.8	46.7	33.3	28.525
28 °C	55	73.7	62.5	62.5	63.425
			***nos*-Cas9**		
18 °C	63.2	65	50	55	58.3
25 °C	65	73.3	61.9	80	70.05
28 °C	65	68.4	63.2	70.6	66.8

Supplementary Table 4

% of flies with mosaic eye					
			***nos*-BE3**		*average*
18 °C	0	0	0	0	0
25 °C	0	0.04	0	0	0.01
28 °C	0	0	0.11	0.11	0.055
			***nos*-ABE7.10**		
18 °C	0	0	0	0	0
25 °C	0.08	0	0.14	0	0.055
28 °C	0.16	0.2	0.93	0.18	0.3675
			***nos*-Target-AID**		
18 °C	0.6	0.78	0.37	0.6	0.6
25 °C	0.13	0.3	0.94	1.2	0.6425
28 °C	2.18	1.16	1.34	1.87	1.6375
			***nos*-Cas9**		
18 °C	1.43	1.15	1.53	1.32	1.3575
25 °C	1.68	2.58	1.78	2.84	2.22
28 °C	3.42	2.5	1.75	2.79	2.615

Supplementary Table 5

% of vials (= crosses) with mosiac flies					
			***nos*-BE3**		*average*
18 °C	0	0	0	0	0
25 °C	0	5	0	0	1.25
28 °C	0	0	13.3	12.5	6.45
			***nos*-ABE7.10**		
18 °C	0	0	0	0	0
25 °C	10	0	12.5	0	5.625
28 °C	11.8	15.8	71.4	18.8	29.45
			***nos*-Target-AID**		
18 °C	41.7	41.7	13.3	25	30.425
25 °C	6.3	33.3	53.3	66.7	39.9
28 °C	80	63.2	50	56.3	62.375
			***nos*-Cas9**		
18 °C	42.1	45	50	50	46.775
25 °C	60	73.3	57.1	85	68.85
28 °C	75	63.2	57.9	76.5	68.15

Supplementary Table 6

F2 generation males scored from *vas*-Cas9 and white-4gRNA cross					
Individual cross	White-eyed males	Mosaic-eyed males	Red-eyed males	Total males	% of white-eyed males
1	34	3	12	49	69.4
2	29	2	5	36	80.6
3	34	-	13	47	72.3
4	44	1	2	47	93.6
5	37	4	19	60	61.7
6	30	3	15	48	62.5
7	28	7	26	61	45.9
8	38	1	8	47	80.9
8	28	2	4	34	82.4
10	54	0	6	60	90.0
11	31	5	17	53	58.5
12	17	3	8	28	60.7
13	40	2	12	54	74.1
14	28	2	30	60	46.7
15	37	2	12	51	72.5

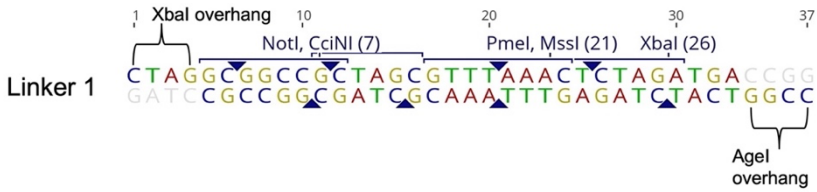


Fig. S1 Details of the linker used for cloning base editor constructs with highlighted restriction sites.

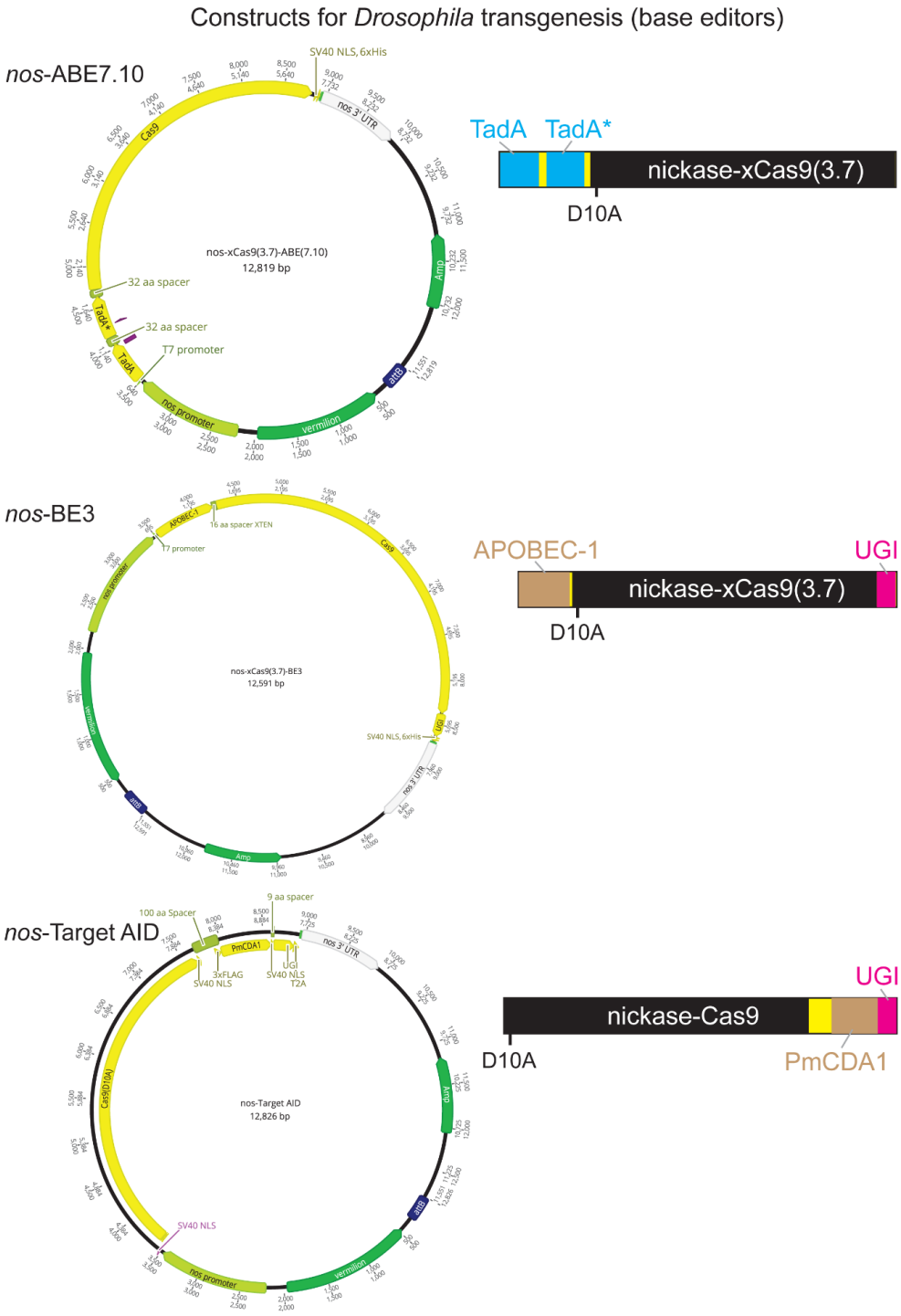


Fig. S2 Constructs encoding three base editors (left) and schematic depiction of the encoded protein (bar on the right).

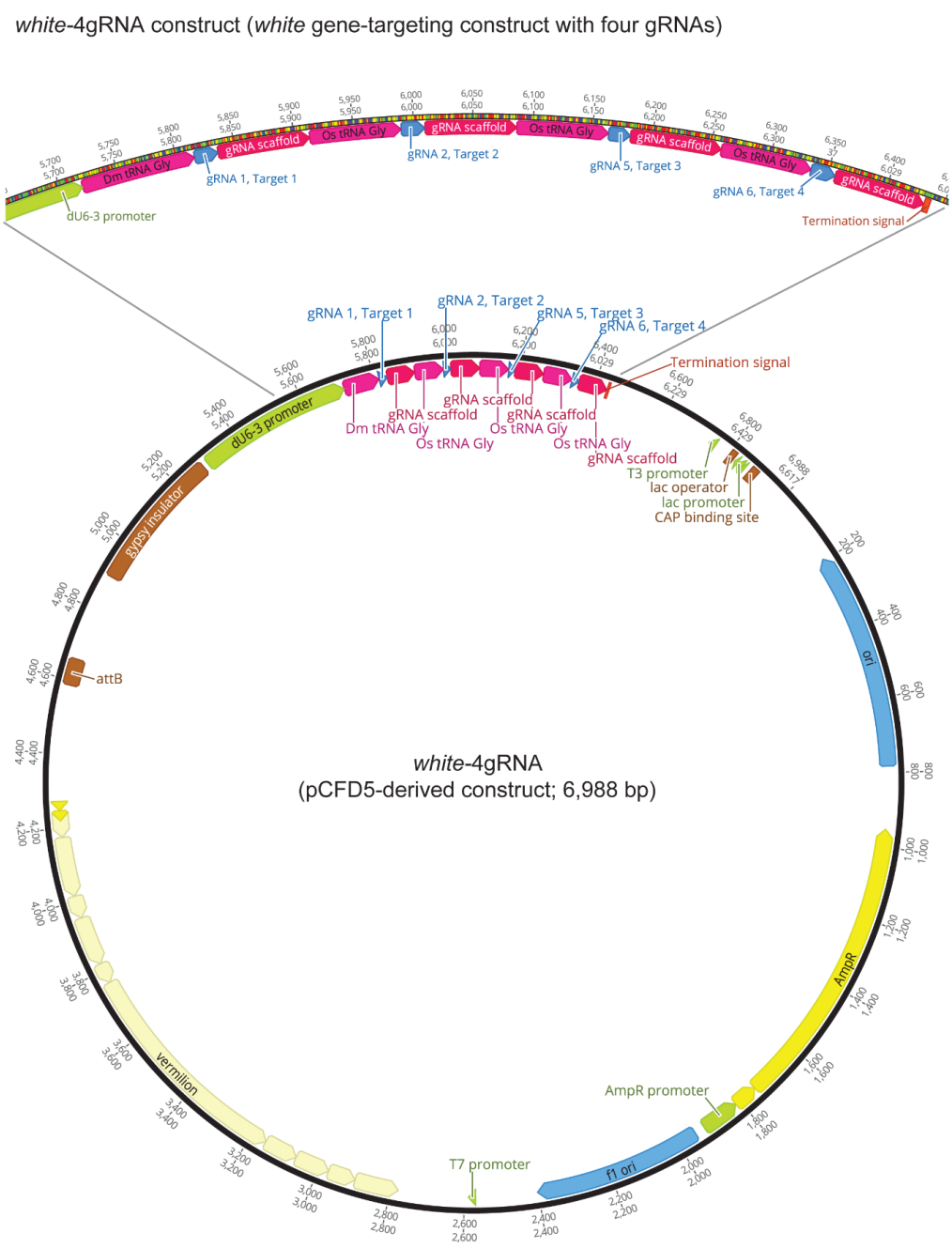


Fig. S3 Multiple gRNA plasmid targeting the *white* gene.

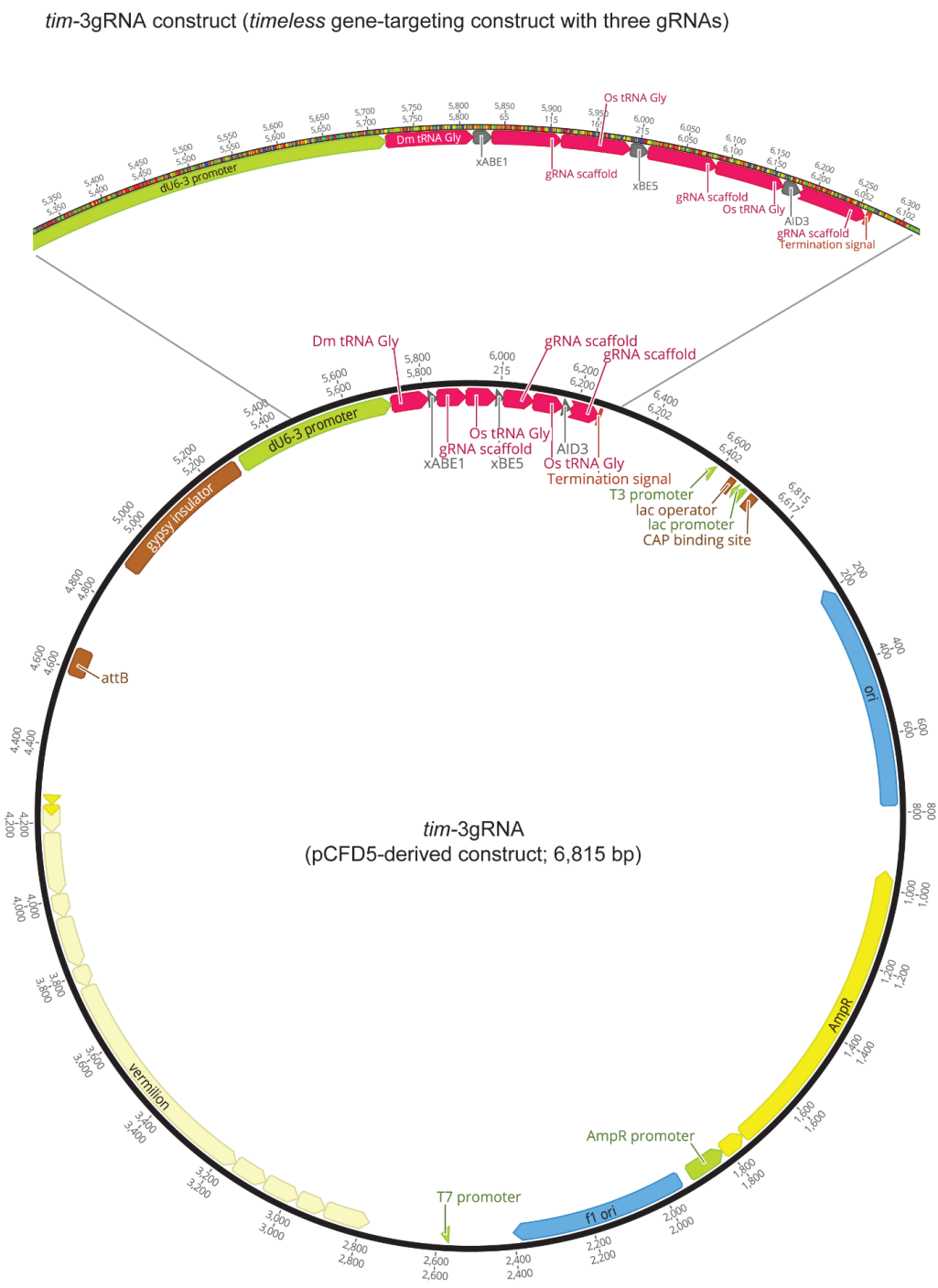


Fig. S4 Multiple gRNA plasmid targeting the *tim* gene.

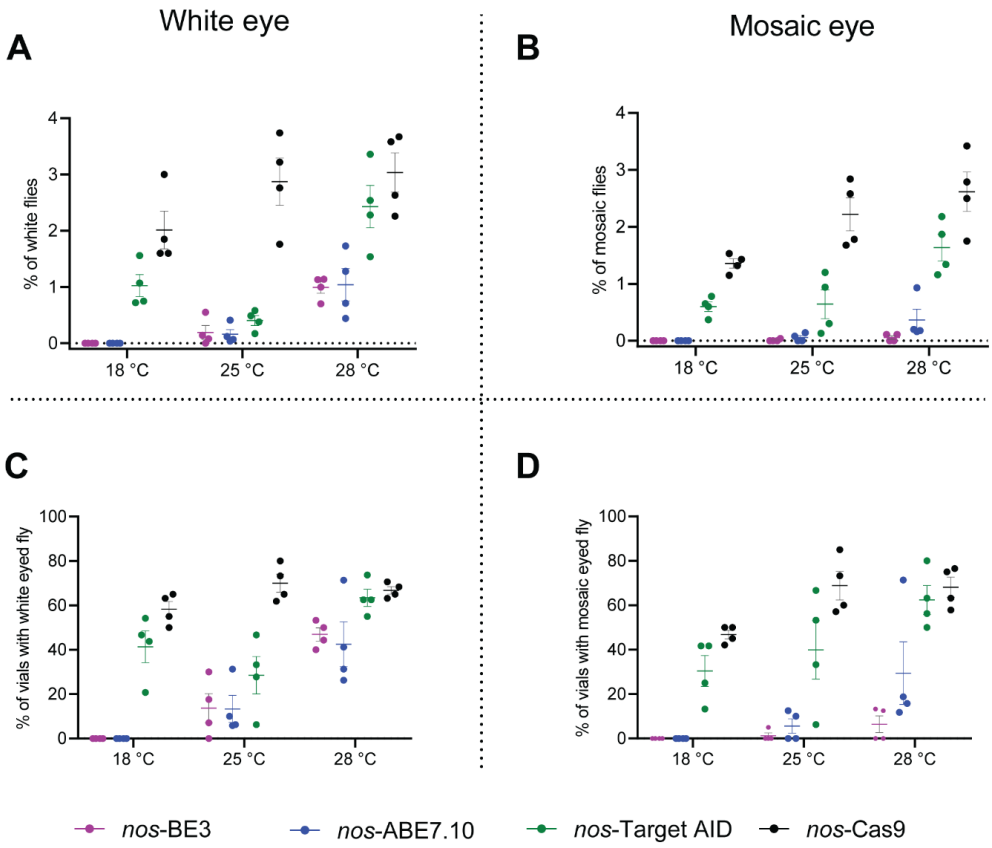


Fig. S5 Base editing efficiency where each of four biological replicates is depicted.

Fig. S6 DNA sequences of the *white* gene mutated by Target-AID.

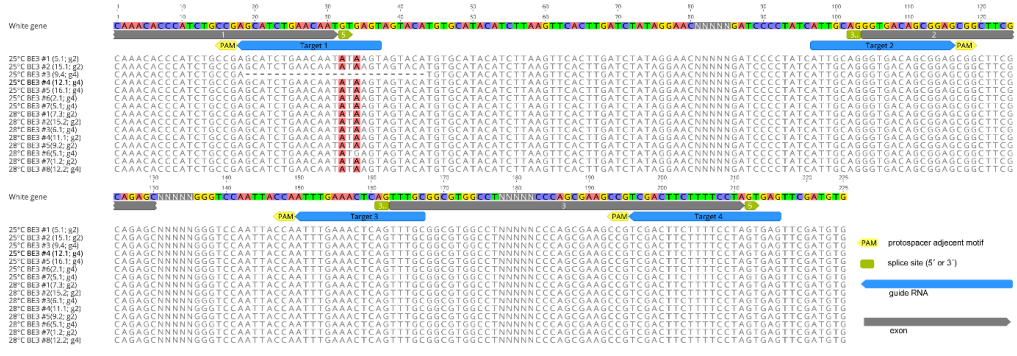


Fig. S7 DNA sequences of the *white* gene mutated by BE3.

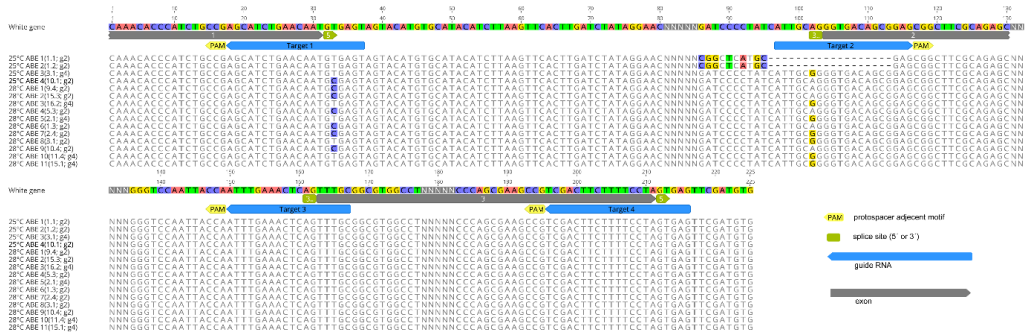


Fig. S8 DNA sequences of the *white* gene mutated by ABE7.10.

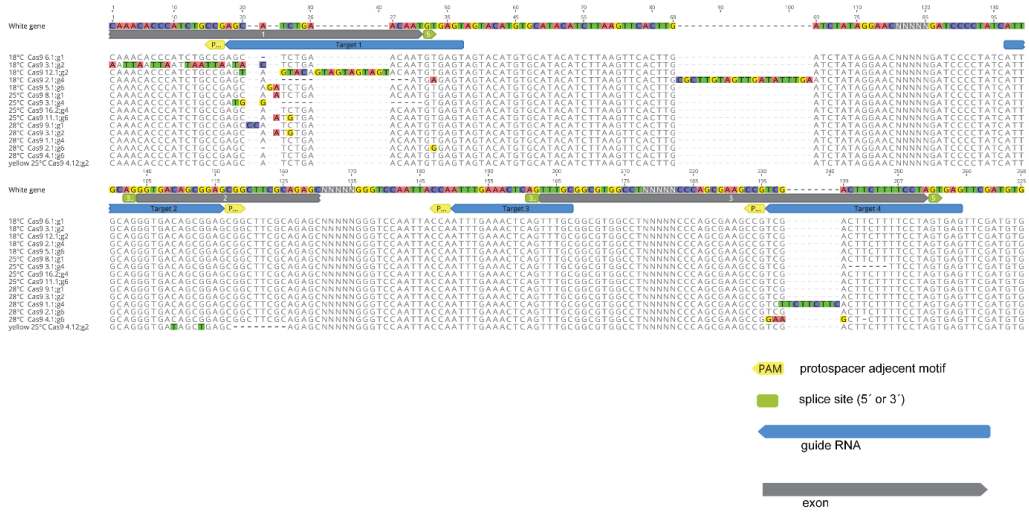


Fig. S9 DNA sequences of the *white* gene mutated by *nos*-Cas9.

Fig. S10 Splice site prediction in the *white* gene in wild-type flies and mutants with white and yellow eyes.

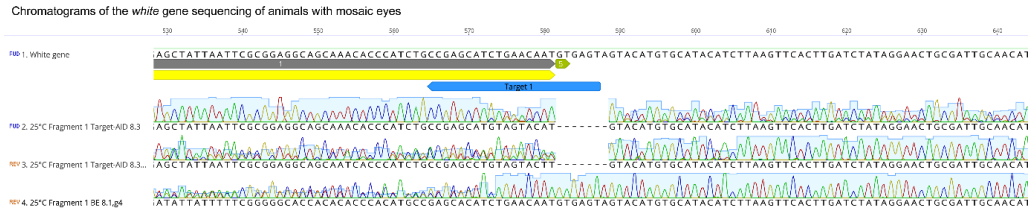


Fig. S11 DNA chromatogram of male with mosaic compound eyes. In the upper chromatogram (sequenced with the forward primer, FW, that is from the left in the scheme), overlapping peaks indicate different frameshifts in the DNA templates. In the bottom two chromatograms (both sequenced with the reverse primer, REV, that is from the right in the scheme), the overlapping peaks on the left are clearly visible. LinesLine #2 and #3 are forward and reverse sequencing reactions from the identical PCR product (identical mosaic male).

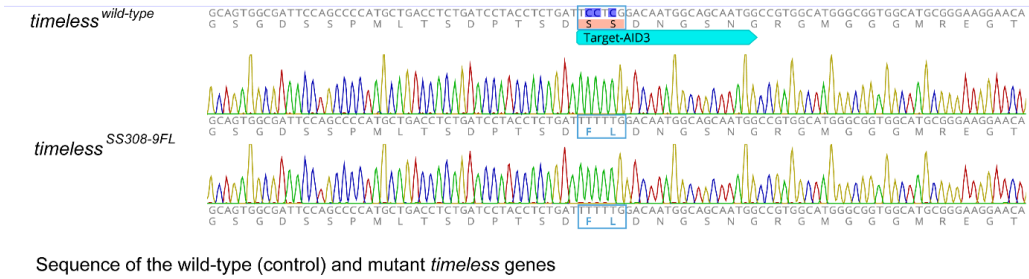


Fig. S12 DNA chromatogram of the *tim* mutant. Blue boxes highlight the change in the DNA and predicted protein sequences. The blue horizontal arrow indicates the position of gRNA.

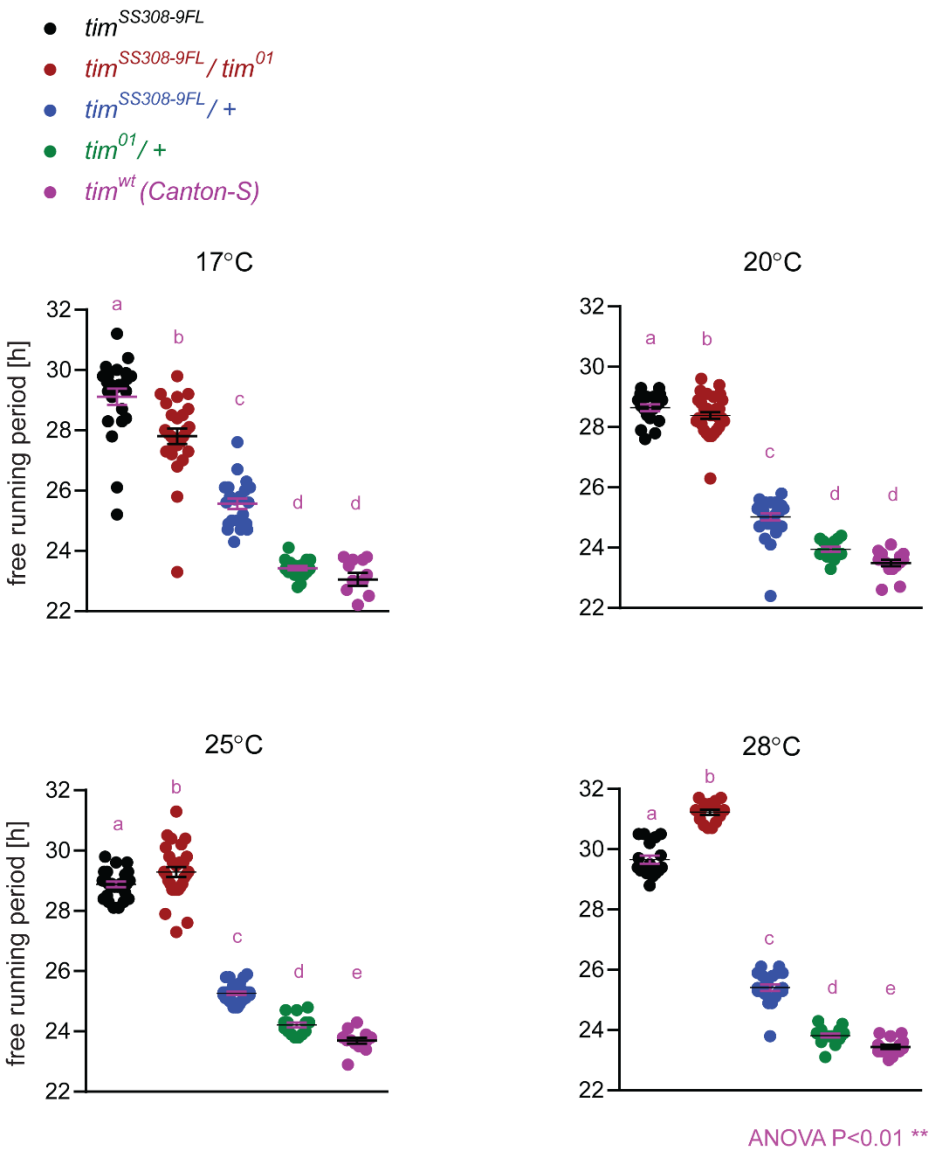


Fig. S13 Comparison of FRPs among lines at each temperature. Bars represent the mean ± SEM and magenta small alphabet letters represent the variance with P<0.01 significance.

Part 3

Unique *Drosophila timeless* mutant with big deletion and severe temperature compensation defect

Singh, S., **Thakkar, N.**, Bullo, E., Parameswaran, A., Rosato, E., & Dolezel, D.

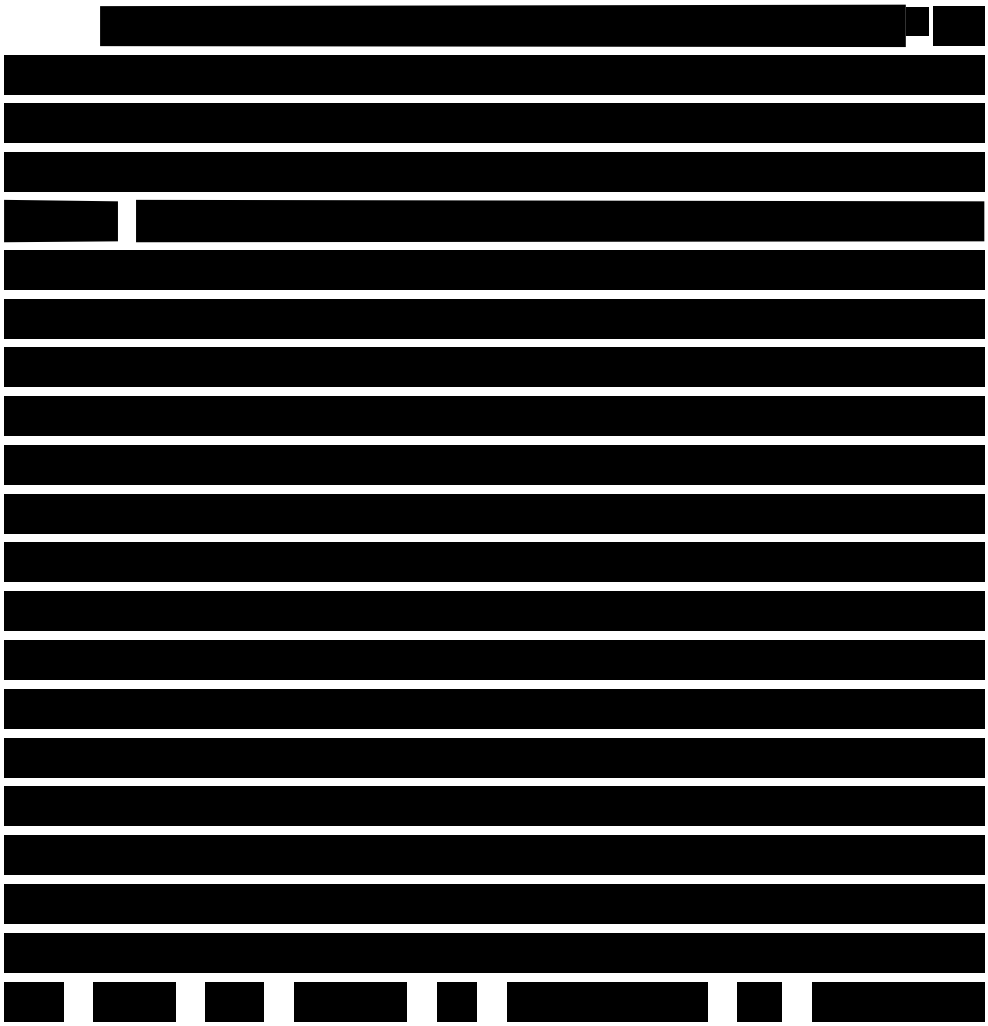
(***unpublished results***)

This thesis focused on addressing two crucial aspects of circadian biology : (1) temperature compensation property and, (2) molecular evolution of clock genes/proteins. Additionally, for the advancement of the scientific research and knowledge, the molecular tools need to be upgraded. Therefore, this thesis also focused on testing the CRISPR base editing tool in *Drosophila* which aided us in creating precise substitution mutant with circadian impairment and will also help in doing controllable DNA manipulations in clock genes in future. Mainly, the above-mentioned aspects were studied here by creating and functional analyzing different mutants of core clock genes such as *doubletime* and *timeless*.

Light and temperature are the two most crucial environmental cues that entrain circadian oscillations (Zeng et al., 1996; Roenneberg and Foster, 1997; Glaser and Stanewsky, 2005; Buhr et al., 2010). Interestingly, while temperature entrains the circadian oscillations, the period of these oscillations is temperature insensitive (Pittendrigh, 1954; Hastings and Sweeney, 1957; Leloup and Goldbeter, 1997; Bodenstein et al., 2012). Despite the scientific efforts in the past decade, the overall mechanism of the temperature compensation of the circadian clock remains poorly understood. We explored the temperature compensation property of the clock in Part 1, Part 2, and Part 3 of this thesis.

In Part 1, we created a mutant of DBT that had a defect in the temperature compensation of the free-running period. Specifically, this heterozygous DBT^{K224D} mutant had a faster running clock at lower temperatures and slower running clocks at higher temperatures. Lysine224 is the important residue that forms the anion binding site 1 in CKIδ and mutation of this site results in impaired phosphorylation of primed substrates (PER FASP2 cluster) compared to unprimed substrates (PER phosphodegron)(Philpott et al., 2020, 2022, 2023; Narasimamurthy and Virshup, 2021). K224D mutants have also been

described for mouse CKIδ and are known to have impaired temperature compensation of the circadian clock (Shinohara et al., 2017). However, mouse CKIδ K224D mutants have slower-running clocks at faster temperatures and faster-running clocks at higher temperatures (Shinohara et al., 2017). It is quite interesting that the modification of the same residue (K224) had a different impact on the temperature compensation in *Drosophila* and mice. Our findings highlighted the lineage-specific differences and complemented the previous findings of the irreplaceability of *Drosophila* DBT with mammalian CKIε despite sequential and functional similarities (Sekine et al., 2008).



With the enhanced understanding of the genetic determinants of the clock, the chronobiology field is shifting more towards understanding the biochemical and structural aspects of the protein. While protein crystallization and modelling are the key components for identifying and understanding the structure, the functional aspects can only be understood by systematically manipulating the coding region of the gene. Therefore, the necessity of creating new mutants remains. Obviously, given the situation, the current requirement is the precise modification of the nucleotides in the particular genes.

In Part 2, we tested two cytosine base editors (BE3, Target-AID) and one adenine base editor (ABE7.10) in the *Drosophila* model system for precise editing of germline DNA. Mainly, to identify the efficiency of these base editors, we targeted the splice recognition sites of the white gene located on the X chromosome rendering easy detection of mutation in male flies. Notably, this project commenced at the beginning of 2019, when no other study had reported the use of a base editor in the *Drosophila* model system. Although during our effort, one study surfaced in 2021, addressing cytosine base editing in somatic cells of *Drosophila* (Marr and Potter, 2021) and another study came out in 2023 which addressed somatic and germline editing using two different cytosine base editors (Doll et al., 2023). We found that base editing is temperature dependent in *Drosophila* with the highest editing at higher temperature and a similar finding was reported by Doll et al. (Doll et al., 2023). Unexpectedly, the efficiency of base editing in our case was suboptimal compared to the efficiency obtained by Doll et al. at 28°C (0.9-2.4% compared to 70-95%)(Doll et al., 2023). The underlying reason behind

the drastic difference in efficiency seen in both studies is the use of different versions of base editors (we tested the earliest developed base editor version while Doll et al. used an engineered version with improved efficiency), the difference in the promoters (we used *nanos* promoter for germline expression while Doll et al. used *actin5c* promoter for constitutive expression in all cells) and the dosage of NLS (Doll et al., 2023).

Apart from these, using the Target-AID cytidine base editor, we made the allele of *timeless (tim$^{SS308\text{-}9FL}$)* where two serines were modified to phenylalanine and leucine in TIM. The circadian clock of *tim$^{SS308\text{-}9FL}$* mutants was disrupted with a longer free-running period of about 29 hours. Interestingly, as hemizygote, *tim$^{SS308\text{-}9FL}$* flies showed defect in the temperature compensation. While it is difficult to figure out the reason behind the defect in temperature compensation of *tim$^{SS308\text{-}9FL}$* mutants, the lengthening of the free-running period can be possibly explained. Nearly all *tim* mutants identified in the serine-threonine-rich region (S-T region) have impaired clocks and longer periods (Rothenfluh et al., 2000; Top et al., 2016; Singh et al., 2019). Top et al. in their study (comprising *tim* transgene mutants with 2-3 serines in S-T region converted to alanine or aspartate) proposed a mechanism in which SGG phosphorylates TIM bound to PER and subsequently, additional phosphorylations are done by CK2 resulting in nuclear localization of PER-TIM complex (Top et al., 2016). The recent CRY-TIM structure paper further clarifies the regulation of nuclear entry of TIM wherein they suggest that the phosphorylation cascade in the ST-rich region changes the conformation of TIM, thereby permitting the proper binding of importin-α1 and the nuclear localization (Lin et al., 2023). Perhaps, the longer period of *tim$^{SS308\text{-}9FL}$* mutants results from the delayed nuclear accumulation of TIM$^{SS308\text{-}9FL}$ protein. Moreover, considering the cytoplasmic-nuclear shuttling involved, the temperature compensation defect of *tim$^{SS308\text{-}9FL}$* hemizygotes may be possibly associated with the

similar phenomena of the temperature compensation defects seen in *tim* mutants that have altered NES. Possibly, future studies will clarify this hypothesis.

Significant progress in understanding TTFL in circadian clocks happened through comparative studies in *Drosophila* and mammals (Allada et al., 2001; Glossop and Hardin, 2002). However, to understand the evolution of the clocks, a larger dataset comprising diverse species is required. Insecta is a class with sufficient diversity where each group came up with its distinct mechanism of surviving and coping with changing environments over the course of years. Moreover, in the era of the sequencing revolution, genomic and transcriptomic data of diverse species are piling up in the NCBI database (Misof et al., 2014; Johnson et al., 2018; Kawahara et al., 2019; McKenna et al., 2019; Wipfler et al., 2019). Therefore, in Part 1, we studied the evolution of DBT and its mammalian homologs in insects and representative deuterostomain species. We systematically compared not only DBT and its mammalian homologs but also other core clock proteins of 55 diverse species (comprising 20 insect orders and 9 deuterostomain classes). Such systematic phylogenetic studies are crucial in identifying the similarities and differences in the protein that emerged during evolution, thereby enabling to identify and target selected regions. The ideal example is Lysine224 residue which is highly conserved in DBT and its homologs in all compared species. However, the modification of the same residue in *Drosophila* and mouse resulted in differently impacted temperature compensation of the clock. Notably, the residues in the vicinity of K224 are diverged in Diptera and form a distinct motif (NKRQK). Based on recent reports of the interaction of DBT with BDBT in *Drosophila* (Fan et al., 2013; Venkatesan et al., 2015; Nolan et al., 2023), when we checked the presence of BDBT in all these species, it was found only in Diptera. Although this correlation needs further verification by doing functional experiments in dipteran species, it illustrates how systematic

comparison may point to a unique and lineage-specific protein modification. Such phylogeny needs to be explored for circadian genes/proteins of deuterostomian classes with a focus on mammals which may aid in understanding and identifying the basis of circadian disorders in humans.

To conclude, this work comprised three distinct parts (Part1-3) that related to the understanding of the broader problematics in the field of circadian biology such as temperature compensation, the necessity of precise functional mutants of clock genes and clock evolution. The altered temperature compensation of *Drosophila* DBT K224D mutation helped us address the lineage-specific differences and [redacted] On the other hand, base edited *timeless* allele ($tim^{SS308\text{-}9FL}$) complements the proposed model of nuclear entry regulation of TIM and perhaps has a potential connection with the temperature compensation mechanism due to its involvement in nuclear localization.

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Nirav Thakkar
niravthakkar26@gmail.com
www.linkedin.com/in/nirav-thakkar-41103166

EDUCATION

Doctor of Philosophy **2017- present**
Molecular Biology and Cell Biology and Genetics, University of South Bohemia, Ceske Budejovice, Czech Republic

Master of Science **2011-2013**
Life Science, Gujarat University, Ahmedabad, India

Bachelor of Science **2008-2011**
Biotechnology, Gujarat University, Ahmedabad, India

Research Internship

University of Leicester, UK **Feb 2022 – July 2022**
21 weeks stay in the laboratory of Dr. Ezio Rosato to get trained in microdissection of *Drosophila* brain, immunohistochemistry, confocal analysis and perform experiments on a collaborative project.

GRANT AND AWARDS

Travel Award for Conference **2022**
Grant Program Biology Centre CAS, European Biological Rhythms Society Congress, Switzerland

Travel Award for Research Stay **2021**
Grant Agency of Jihoceska University, Research stay at Dr Ezio Rosato's lab, UK

Travel Award for Research Stay **2021**
Grant Program Biology Centre CAS, Research stay at Dr Ezio Rosato's lab, UK

ERASMUS+ Mobility fellowship **2019**
Research stay at Dr Ezio's lab, UK

Travel Award for Conference **2019**
Grant Program Biology Centre CAS, GRC Chronobiology, Spain

Two-year Individual PhD student grant **2019-2020**
106/2019/P, Grant Agency of Jihoceska University, Project: Factors responsible for altering free-running period across the geographic variability in *Pyrrhocoris apterus*

One-year Individual PhD student grant **2018**
079/2018/P, Grant Agency of Jihoceska University, Project: Functional analysis of circadian clock genes in *Drosophila melanogaster*

CONFERENCE PRESENTATIONS

Thakkar, N., Hejzlarova, A., Brabec, V., and Dolezel, D. CRISPR Base-editors: Highly precise and scar-proof genome-editing tool to generate unique point mutants and explore clock genes in *Drosophila melanogaster*. European Biological Rhythms Society. **Poster presentation** delivered at XVII European Biological Rhythms Society Congress, Zurich, Switzerland, 24-28 July, 2022

Thakkar, N., Wu, BC., and Dolezel, D. Genetic factors responsible for altering free-running period (FRP) across the geographic variability in *Pyrrhocoris apterus*. Gordon Research Conferences. **Poster presentation** delivered at Gordon Research Conference on Chronobiology, Castelldefels, Spain, 22-28 June, 2019

PEER-REVIEWED PUBLICATIONS

Thakkar, N., Hejzlarova, A., Brabec, V., & Dolezel, D. (**2023**). Germline Editing of Drosophila Using CRISPR-Cas9-based Cytosine and Adenine Base Editors. The CRISPR Journal. DOI: 10.1089/crispr.2023.0026

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niravthakkar26@gmail.com

Functional analysis of circadian clock genes in *Drosophila melanogaster*
Ph.D. Thesis, 2024

Printed in the Czech Republic by Typodesign
Edition of 5 copies

University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 1760
CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201
www.prf.jcu.cz, e-mail: sekret-fpr@prf.jcu.cz