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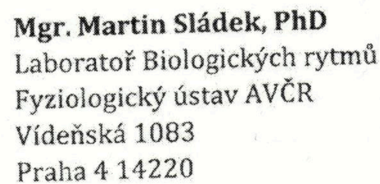

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The PhD thesis of Samarjeet Singh focuses on a little understood aspect of circadian clock, namely its temperature compensation mechanism that allows the clock to run with constant period at different environmental temperature. The defendant used modern molecular genetic tools such as CRISPR/Cas9 editing, to modify selected regions of a crucial fruit fly clock gene *timeless* and subsequently observe the rhythmic behaviour at different temperatures and light regimes. The core of the work is formed by smart hypothesis-driven approach to study the topic and contains interesting data that are not simply descriptive, but bring a novel insight into possible mechanisms of temperature compensation. The dissertation is written in good English with just a few mistakes, but check usage of first person singular vs. plural vs. passive. The introduction is perhaps too brief with just 15 pages, but overall nicely written and introduces most concepts. I missed more details on molecular mechanisms of alternative splicing.

Minor comment:
Page 16/paragraph 2 – CRY/light-mediated degradation of TIM is nicely explained, however the paragraph ends somewhat misleadingly, suggesting that this is necessary to generate rhythms - "Thus, the clock resets to start an entire new cycle which takes about 24 hours to complete and hence generates circadian rhythms." However, the clock functions perfectly well in constant darkness or without CRY. The sentence should be worded more precisely.

Chapter 1
This is defendant's only published paper so far. It is included as a part of the dissertation. It describes 20 newly generated mutations that affect the ability of the fruit fly to compensate its clock speed for the environmental temperature increase. It also identified an entirely new region of TIM protein that is important for temperature compensation. The paper itself involved a huge amount of work. Including the supplement, it contains 16 multi-panel figures. Defendant was responsible for both the design of the study and writing of the paper, and performed majority of the time-consuming experiments, that included CRISPR-mediated generation of tens of novel mutations in *tim* gene, corresponding behavioural and molecular screening, *in silico* analyses, etc. The work is published in a solid peer-reviewed open-access journal where it, in my opinion, punches well above its weight. It seems genuinely interesting and well written, though that is to be expected from this excellent lab.

Chapter 2
Nice work in progress, that demonstrated importance of alternative splicing of *tim* gene for drosophila clockwork. Using CRISPR, author first mutated stop codons in retained intron, thus creating long mutated isoforms of *tim*, that often resulted in different period at higher temperatures. Then he showed, that full length isoform of *tim* is necessary for the clock to function, as it can't be replaced by truncated *tim* isoforms. The work just needs statistics and more in-depth discussion before publication.

Comments:
Statistical analysis of free running period data seems to be missing. Some claims are therefore unsubstantiated, for example on page 85, that "mutant F104 clock runs faster even at low temperatures".

Fig 3 and Fig 5 show the same data of FRP of homozygous flies, just expressed differently – it would be better to use the space to show at least a few heterozygous mutants instead and demonstrate the supposed dominant effect of these mutations the author wrote about on page 85.

Author sometimes uses the term "clock advance", which is associated with phase and suggests a fixed time reference point (such as in "advanced evening peak of activity on LD"), when actually referring to experiments in constant darkness. Instead, I would suggest to use for example "shorter period" or "faster clock" when referring to free running experiments in constant conditions.

Chapter 3
Extremely interesting result that was achieved using custom made flies from a commercial supplier that allow highly accurate genetic engineering of *tim* gene that lacks specific exons coding CRY interaction domain but still retains the cytoplasm localization domain. Surprisingly, the mutant flies were rhythmic in constant light depending on the environmental temperature, suggesting a temperature-dependant interaction of TIM protein with other clock components. This insight and the opportunity the model offers for future detailed examination of the *tim* function serve as an exciting conclusion of the work.

I gladly recommend the dissertation for defence.

Questions:
Q1: Do you know whether the mutants entrain efficiently to temperature cycles in comparison with WT? If not, are there any known fly mutations that affect temperature entrainment?

Q2: Is it known whether SGG or CK2 is necessary for T-compensation? Is there any clue as to what kinase might be responsible for phosphorylation of the putative P-site on TIMs nuclear export signal?

Q3: Regarding your *tim* mutants in chapter 1, all of them had longer period in higher temperature. Increase in temp should speed up most molecular mechanisms affecting period, such as transcription rate, RNA processing, nuclear translocation, protein degradation, etc., so I

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would expect the clock to be quicker and period shorter. Would it be possible to talk about temperature overcompensation rather than deficit in compensation?

Q4: Are there any known *tim* gene polymorphisms between fruit flies living in different climate zones?

Q5: In mammals, there is temperature cycle-regulated alternative splicing of many genes including transcription factor *Tbp*, but likely not of any core clock gene. Are there any known temperature sensitive alternative splicing events outside the *tim* and *per* UTR in *Drosophila*?

Two more optional speculative questions:

Q6: Can you speculate why mutation F-104 in chapter 2 prolonged the period in high temperature, while all the other mutations, some of which seemed very similar, resulted in shorter period in higher temperature?

Q7: Do you think that TIM conformation could be temperature sensitive and that it could serve as the temperature sensor?

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V PRAZE 4. 6. 2020

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12/06/2020

PhD Thesis: Functional analysis of circadian clock gene *timeless* in temperature compensation mechanism.
Candidate: Samarjeet Singh, School of Doctoral Studies in Biological Sciences, Faculty of Science. University of South Bohemia in České Budějovice
Examiner: Dr Ezio Rosato, Department of Genetics and Genome Biology, University of Leicester, Leicester, UK.

Examiner Report

The broad topic investigated by the thesis is the regulation of the circadian clock in the fruit fly *Drosophila melanogaster*, a popular model system in the life sciences with a great arsenal of molecular tools available. This includes gene editing by CRSPR/Cas9, a technique repeatedly and successfully used in this work.

The research focusses on the investigation of temperature compensation a 'mysterious' property of the circadian clock that prevents the measurement of time being dependent from the temperature of the environment, a real threat for poikilotherms. The 'enigma' is that as temperature changes so does the speed of biochemical reactions. The implication is that more than one reaction must be at stake with opposing, compensatory effects on the final outcome. We do not know the identity of those reactions. Additionally, 'coupled', compensatory processes could be at play in the same cell – according to the classic cell-autonomous model of the clock – or in different cells. The latter hypothesis is based on a recent interpretation of the clock that leverages the 'network' organisation of the nervous system (where the clock in charge of behaviour resides). It assumes that interactions among clock expressing neurons result in 'emerging' properties that do not otherwise manifest in any single cell. On this premise, it follows that temperature compensation, in addition of being an interesting phenomenon *per se*, is a useful paradigm to gauge the point of contact between the two different, but not alternative, hypotheses on clock organisation. Thus, a first conclusion is that the topic of this thesis is extremely relevant for chronobiology and beyond. Moreover, this work is important as it aims to contribute tools, observations and

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analyses to inform on how neurons employ homeostatic mechanisms to produce behaviour in a timely fashion.

To realise his aim the candidate embarked on CRISPR/Cas9 mutagenesis of the *timeless* (*tim*) locus. The *tim* gene is one of the canonical components of the circadian clock, it encodes a transcription regulator that together with a partner protein called PERIOD (PER) intervenes in orchestrating rhythmic transcription in cells. The mechanism is based on a negative feedback loop such that PER and TIM rhythmically repress their own transcription and that of additional clock-controlled genes as part of a generation-maturation-degradation-generation-[...] cycle that involves extensive post-translational regulation of the two proteins and lasting about 24h.

In the first result chapter (published paper in Frontiers) the candidate mutagenized several regions of *tim* to identify temperature-dependent variants that may shed light on the temperature compensation mechanism. Target regions were chosen on the basis of amino acid sequence conservation across evolution and on the knowledge of conventional mutants, such as tim^{ritsu} and tim^{blind}, affecting temperature compensation. This work resulted in the generation of 113 arrhythmic (under constant darkness, DD) variants and in the production of about 20 rhythmic mutants affected in period length. Of these, many showed temperature-dependent periodicity, especially those mapping in proximity of the two aforementioned classical mutations.

In the second result chapter the candidate applied CRISPR/Cas9 mutagenesis to investigate alternative splicing affecting a defined region of *tim*. In the *tim* locus, three introns undergo temperature-dependent alternative splicing generating the variants *tim-cold*, *tim-short&cold* (*tim-sc*) and *tim-medium* (*tim-M*). *tim-cold* and *tim-sc* are more common at low (below 25 °C) temperature while *tim-M* is more abundant under warm conditions (25 °C and above). As a consequence, the relative abundance of *tim-L*, the main splicing isoform, changes in a temperature-dependent fashion. Focussing on *tim-M* the candidate targeted the intron retained at high temperature to remove the two stop codons resulting from the read-through. Twelve mutants were obtained lacking both stop codons. They were rhythmic in DD with some showing various degrees of temperature dependence in period-length. To verify further the role of TIM-M, the candidate introduced a stop codon in the exon immediately downstream the retained intron, as to obliterate production of the main (TIM-L) variant. In agreement with published work, TIM-M was unable to sustain endogenous rhythmicity.

In the last result chapter the candidate characterised *tim-attP*, a mutant engineered by CRISPR/Cas9 and missing exons 10-14. At the protein level such

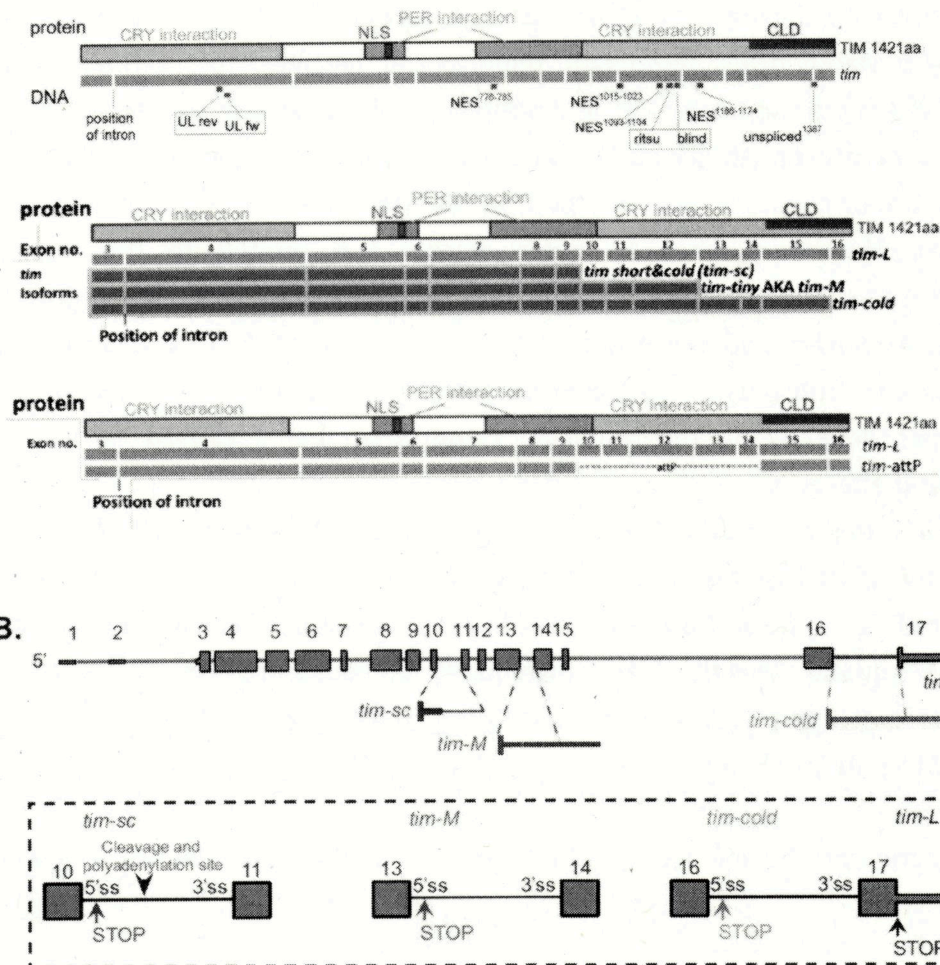
variant is missing the majority of the second (C-terminal) CRY interaction domain, while maintaining other important regions and motives. Interestingly, such mutant was rhythmic under DD and showed a progressively longer period at higher temperatures. Additionally, and even more surprisingly, the mutant was rhythmic under constant light, a condition that generates arrhythmicity in wild type flies. Moreover, when in heterozygosis and limited to the highest temperature tested (28 °C) the rhythmic flies segregated into two distinct groups, one with a shorter and the other with a longer period. Rescue experiments (reinstating the integrity of the *tim* locus in the mutant) demonstrated that such phenotypes depends on the mutation rather than being caused by genetic background effects.

In summary, the thesis describes a large and important experimental effort, it is well presented and well written with only minor errors that do not compromise understanding. Some of the work presented is published, the remaining part is of publishable quality. This thesis is of high quality and compares very well with the standards that apply in my home institution. I recommend that the thesis is admitted to public defence.

In preparation for the defence I would ask the candidate to consider the following queries.

1. Across all chapters the nomenclature of exons/introns you used differs from that adopted by other authors of comparable work.

For instance, the first three figures below comes from Chapter1, 2 and 3, respectively. Beneath are corresponding figures from Martin Anduaga et al. eLife 2019;8:e44642. Could you please comment?



2. I could not find cited a reference reporting the mapping of the CRY interaction domains in TIM

3. In your figures you report TIM as a 1421 aa long protein, which corresponds to the l-TIM variant. However, with the exception of *tim-attP* (derived from *ls-tim*), it is not clear which variant you targeted for mutagenesis.

4. It is not clear whether, before analysis, you backcrossed CRISPR/Cas9 mutants to eliminate (as much as possible) random mutations deriving from non-specific Cas9 activity.

5. In chapter 1 you identified several mutants whose period is temperature-dependent. For many of them temperature seems to affect rhythmicity *per se*. In other words, with increasing temperature the number of rhythmic flies decreases. This seems to become even more pronounced in a tim^0 background, supposedly when reducing protein content by half. Could it be that the phenotype of these

mutants is more a reflection of the growing instability of the protein at higher temperature rather than being informative about a specific temperature compensation defect?

6. In chapter 1 you put great emphasis on the possibility that temperature-dependent mutants might have compromised NES signals. Is there a theoretical reason why you would expect a temperature compensation phenotype from compromising nuclear export of TIM?

7. In chapter 2 you investigated what role TIM-M might have in rhythmicity and you found none. This observation is in agreement with the results that Martin Anduaga et al. found using a luciferase reporter. Are these findings really surprising considering that a general surveillance mechanism called nonsense-mediated mRNA decay (NMD) eliminates mRNA carrying stop codons that are far away from cleavage and polyadenylation sites? Following from this, have you considered testing the splicing and the mRNA stability of your mutants?

8. At the beginning of your work, why did you not consider generating first a version of *tim* encoding for a tag and then starting mutagenesis? You would have been able to consider protein expression and stability and to ascertain the subcellular localisation of each mutant.

9. In several places in your thesis you talk about and 'advanced clock' when you actually mean a faster ticking clock (for instance, pg.85). What is the difference between 'advanced' and 'faster'?

10. On page 85 you state: "It thus, suggests that deletion of stop codons in the retaining intron resulted into a longer isoform of *tim*, even longer than the canonical *tim-L* isoform, which adds more TIM in cell thereby advancing the clock". Could you explain the meaning of such a sentence?

11. The *tim-attP* mutant is rhythmic under LL. You stated that it was produced from the ls-tim variant. You are also aware that *ls-tim; jet*^{c} flies are rhythmic under LL (Peschel et al., 2006, PNAS, 46:17313). It would seem logical to test which *jet* allele these flies are carrying. Did you?

12. The *tim-attP* mutant shows loss of temperature compensation. Have you considered testing the other temperature compensation mutants under LL

conditions? Perhaps even *tim-sc*, since it partially rescues *tim*^{0} in DD (Martin Anduaga et al., 2019)?

13. In DD at 28 °C, *tim-attP/+* and even more so *tim-attP/tim*^{0} flies segregate in two groups with different period. Do those flies show a unique rhythm or multiple rhythms and you recorded only the more significant one?

14. How do you think the experiments you initiated should progress if you had more time to carry out this research?

Yours faithfully,

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Reader in genetics

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