UNIVERSITY OF OSTRAVA
FACULTY OF SCIENCE

TO WHOM IT MAY CONCERN

Opponent´s report on the dissertation thesis "**Interplay between the tRNA anticodon stem and small ribosomal proteins forming the decoding site during stop codon readthrough**" by Zuzana Čapková (Pavlíková), Faculty of Science, Charles University, Prague

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The thesis deals with a fundamental molecular process taking place in every cell – translation. Elucidation of the general mechanism of translation, also called proteosynthesis, has become one of the major triumphs of molecular biology and has yielded numerous Nobel prizes. Yet, despite decades of extensive research, important unanswered questions remain concerning particular aspects of translation, including mechanistic details as well as evolutionary variation across taxa. In her work, Zuzana Čapková (née Pavlíková) employed cutting edge methods of molecular biology and the possibilities provided by the yeast *Saccharomyces cerevisiae* as a model system to obtain new significant insights into both the mechanisms and evolution of translation. The scope and the ambitions of her research are absolutely appropriate for a PhD thesis in a molecular biology-oriented PhD program, and she has also fulfilled the standard requirement for having at least a part of the results published in peer-reviewed journals. Zuzana has met the latter criterion in a spectacular manner, having one her papers published as a co-first author in *Nature*. With that, my role as an opponent of the thesis is more or less formal. Still, I have critically evaluated the whole thesis and below briefly comment on the two published papers and the submitted manuscript included as annexes while elaborating more on the theoretical background and general discussion that sandwich the core consisting of the research papers.

<u>Original results by Zuzana Čapková</u>
These are reported in three papers, two of them published and one still under consideration by a journal as of the date of writing this evaluation. The thesis describes in detail the specific contribution of Z. Čapková to each paper, which in all cases includes both performing research as well as writing. Zuzana's authorship as such and her position in the authors lists are thus fully justified in all three cases.

The oldest paper, entitled "**Yeast applied readthrough inducing system (YARIS): an *in vivo* assay for the comprehensive study of translational readthrough**" and published in 2019 in *Nucleic Acids Research* is an excellent study providing a systematic dissection of the ability of tRNAs specified by the yeast nuclear genome to support (upon their overexpression) translational readthrough of the near-cognate termination codons. Zuzana is listed as a middle author of this paper and performed the dual-luciferase reporter assays presented in several figures in the paper.

The second paper corresponds to the aforementioned study published in *Nature* in 2023 and entitled "**Short tRNA anticodon stem and mutant eRF1 allow stop codon reassignment**" and equal contribution of Zuzana and two other authors (listed on the first and third position) is indicated. This study builds on findings obtained years ago in my own lab and develops them in a striking direction. Above all, the authors demonstrated that to implement a stop-to-Trp reassignment of the

UGA codon, two different eukaryotes – the trypanosomatid *Blastocrithidia nonstop* and the ciliate *Condylostoma magnum* – have altered their $tRNA^{Trp}_{CCA}$, normally efficiently decoding only the UGG codon, by a mutation loosening the top base pair of the anticodon stem. This structural alteration enhances the ability of the tRNA to decode the near-cognate UGA codon, which is further supported by a mutation in the eRF1 protein decreasing its affinity to UGA, reducing thus the competition with the $tRNA^{Trp}_{CCA}$ in UGA binding.

The third paper included in the thesis is still in a manuscript form, presently being evaluated as a revision of an already reviewed previous version in *Nature Structural & Molecular Biology* (the status confirmed by the corresponding author Leoš Valášek). It is entitled "**Ribosomal A-site interactions with near-cognate tRNAs drive stop codon readthrough**" and Z. Čapková shares the first authorship with another labmate. The key findings reported in the paper are that the ability of certain tRNA to promote efficient stop codon readthrough depends on particular features of their anticodon stem and that interactions of the anticodon stem with certain ribosomal proteins in the A site of the ribosome are critical for the effect. The novelty and significance of the research presented in the manuscript is very high and there are no doubts it will eventually be published in the journal.

General introduction and discussion presented in the thesis

Apart the three papers mentioned above, the thesis consists of a series of additional parts together forming a fairly standard thesis structure. I assume that these parts are original texts by Z. Čapková herself with a minimal input from others and hence I take them as a proxy of her skills in scientific writing. The most substantial of these sections are a brief Chapter 1 ("General introduction"), Chapter 2 ("Current state of knowledge") presenting a broader general review of the area including the research topic of the thesis, and Chapter 6 ("Discussion") reflecting on the main results of Zuzana's research. The total length of these sections and the depth with which the material is presented are absolutely appropriate. Nevertheless, I do have some critical comments to both formal and factual aspects of these parts. Firstly, I have noticed numerous linguistic issues, including typos, missing or incorrectly used articles, grammatical mistakes, questionable word order, incorrect punctuation, or inappropriately used terms. Some inconsistencies hold also for in-text citations, such as including initials of the authors (e.g., "F. H. C. Crick, 1966") in some citations but not others or using a strange formatting "proposed/shown/demonstrated/suggested etc. by (AUTHOR, YEAR)" instead of "proposed/shown/demonstrated/suggested etc. by AUTHOR (YEAR)". These issues make reading of the text slightly less entertaining than it would be if Zuzana paid more attention to details, but in the context of the thesis as a whole they do not truly undermine a generally positive impression the thesis gives to the reader. Concerning the factual content, let me raise some critical comments explicitly:

1. Chapter 1 includes the following passage (page 6): "Interestingly, an astonishing reassignment of all three stop codons to sense codons has recently been observed in four evolutionary very distant organisms, including a parasitic trypanosomatid *Blastocrithidia* (Záhonová et al., 2016). Therefore, another exciting goal of my thesis was to gain more insight into how this organism named *Blastocrithidia nonstop* dealt with this deadly phenomenon by studying specific mutations in the backbone of its $tRNA^{Trp}$ and of its release factor. The result was published in

Nature with me featuring as one of the co-first authors." Putting for the moment aside the fact that the number of organisms exhibiting all three stop codons reassigned as sense codons is not really up-to-date (see another comment below), my major problem is that the "another exciting goal" stated in the text is too ambitious and not really followed by the work presented in the thesis. What is unusual about the genetic code in *Blastocrithidia*, *Condylostoma* and the other organisms with all three stop codons reassigned is the fact that the translation system in these organisms can discriminate the meaning of one or multiple codons depending on their position in the coding sequence, interpreting them as either a sense or a stop codon; e.g. UAA in *Blastocrithidia*. In contrast, the research done by Zuzana and co-workers and ultimately presented in the *Nature* paper aimed at a different question: how UGA is decoded as Trp in these organisms. Indeed, the structural alteration of $tRNA^{Trp}$ enabling the tRNA to read UGA, described by these authors, applies more generally to the eukaryotic translation system and is not restricted to eukaryotes with all three stop codons reassigned or with dual codon meaning as such. This is clearly illustrated by the ciliate *Blepharisma stoltei*, which has UGA fully reassigned as a Trp codon while preserving UAG and UAA as "pure" termination codons, and which (like *Blastocrithidia* or *Condylostoma*) features $tRNA^{Trp}$ with the standard anticodon CCA yet with the topmost anticodon stem base pair loosened (resulting in a "4-bp AS tRNA"; see Swart et al. 2024, eLife 13:RP93502, https://doi.org/10.7554/eLife.93502.1). The molecular mechanism behind the position-specific decoding of one or more stop codons, seen in *Blastocrithidia*, *Condylostoma* and several other eukaryotes, thus remain as poorly understood as it was in 2016 when these genetic code variants were observed for the first time.

2. On page 13, the following statement is presented: "alternative genetic codes have been reported in both nuclear and organellar genomes from bacterial to eukaryotic species". It is highly misleading, as bacteria have neither nuclear nor organellar genomes.

3. Another problematic text is also found on page 13: "Surprisingly, reassignment of all three stop codons has been observed in four organisms so far. Namely, in a trypanosomatid *Blastocrithidia* sp. (Záhonová et al., 2016), in a dinoflagellate *Amoebophrya* (Bachvaroff, 2019) and in cialiates *Parduczia* and *Condylostoma* (Heaphy et al., 2016; Swart et al., 2016)" (see also an analogous statement on page 6 cited above and the sentence staring "Only a few years ago, four organisms were discovered …" on page 37). Putting aside the typo in the term "ciliates", the issue here is that the list of cases with all three termination codons reassigned is outdated and incomplete. Specifically, numerous additional ciliates from classes Karyorelictea, Plagiopylea, and Spirotrichea have been reported to exhibit all three termination codons reassigned as stop codon (while keeping the original translation termination function by at least some of them); see Seah et al. 2022, Peer Community Journal 2:e42, https://doi.org/10.24072/pcjournal.141; McGowan et al. 2023, PLoS Genet. 19:e1010913, https://doi.org/10.1371/journal.pgen.1010913; Chen et al. 2023, Mol Biol Evol. 40:msad064, https://doi.org/10.1093/molbev/msad064. Furthermore, an analogous phenomenon has also been recently (yet well before the submission of the thesis) reported from mitochondria of the protist group Radiolaria (Macher et al. 2023, mBio 14:e0030223, https://doi.org/10.1128/mbio.00302-23.

4. I think there is a mistake in the amino acid residue numbering or the reported identity of the amino acid at the position 4 in the following sentence (page 19): "Moreover, in regards to the rotated hybrid states, structure of the human 80S complex with A/A- and P/E-tRNAs (PDB 6OM7) showed that Val4 might contact positions 27 and 28 of the A-site tRNA". The point is that according to Fig. 7 on the same page, the human eS30 protein has Val at the position 2 but Gly at the position 4.

5. I have multiple problems with the following statement on page 38: "Later, individual screens for more efficient suppressors of $tRNAs^{Trp}$ in *E. coli* (Ortiz-Meoz & Green, 2010; Schultz & Yarus, 1994b) or $tRNA^{Gln}_{CUG}$[M] in *S. cerevisiae* (Kemp et al., 2013) independently of each other discovered that tRNAs with mutations of the top bp in the anticodon stem enhanced stop codon readthrough in comparison to the wild type tRNA. These more potent tRNAs had its anticodon stem shortened from the canonical 5 bp to 4 bp, resembling the *B. nonstop* readthrough-inducing $tRNA^{Trp}_{CCA}$". Firstly, I do not think it is appropriate to write "suppressors of tRNA". Here the suppression means counteracting phenotypic consequences of particular mutations, in the cases cited above specifically the occurrence of an in-frame UAG codon, so the correct wording would be suppressors "of in-frame UAG" rather than "of tRNA". Secondly, in the case of the study by Schultz & Yarus (1994b) the alterations of the anticodon stem that enhanced the suppressor activity concerned a tRNA that was initially mutated in the anticodon itself, having changed the standard anticodon of $tRNAs^{Trp}$, i.e. CCA, to CUG (complementary to the Gln codon CAG rather than to the Trp codon UGG). Hence, the comparison was not made to the "wild type tRNA". Thirdly, according to the data presented by Ortiz-Meoz & Green (2010), loosening by mutation the top base pair of the anticodon stem of $tRNA^{Gln}_{CUG}$[M] alone had a minimal effect on the ability of the tRNA to promote UAG readthrough (they explicitly write "C27A resulted in no miscoding on its own") and the effect of the mutation became apparent only when it was combined with mutations at other positions of the tRNA molecule. Finally, I am not sure if the comparison of the suppressor tRNA variants studied earlier by Ortiz-Meoz & Green (2010) or Schultz & Yarus (1994b) to the "*B. nonstop* readthrough-inducing $tRNA^{Trp}_{CCA}$" is appropriate. In the former cases the effect of anticodon stem mutations concerned the ability of the tRNA to decode UAG via the first codon position wobble (U-G pairing), whereas in the case of the *B. nonstop* $tRNA^{Trp}_{CCA}$ the effect of the anticodon stem shortening is manifested at the third position of the UGA codon pairing with C at the first anticodon position (i.e. a nonstandard A-C wobble). I think apples and oranges are being compared here.

6. I think that the overview of nucleotide positions in the tRNA primary structure that influence translational readthrough as presented in Fig. 9 (page 24) is incomplete. For example, the A-to-C mutation of $tRNA^{Trp}$ at the position 9 has been reported to increase stop codon readthrough (as discussed in Schmeing et al. 2011; a paper cited in the thesis), but the position is not marked in Fig. 9.

7. Given the fact that the second paper included in the thesis prominently features eRF1 and the role of a specific mutation in this protein in the stop-to-sense reassignment in *Blastocrithidia* (and *Condylostoma*), it is somewhat surprising that the review part of the thesis (Chapter 2) does

not pay much attention to eRF1 and does not cover at all the previous numerous reports on various eRF1 modifications demonstrated to influence its ability to recognize different codons and mediate translation termination based on them.

In summary, the thesis by Zuzana Čapková is not flawless, but the problems I have identified are relatively minor and I have absolutely no hesitation to **recommend the thesis for defence**. I expect Zuzana addresses my points listed above at the defence itself, yet to further promote the scientific debate, I encourage her to think about the following extra questions that came to my mind when reading "Conclusions" of her thesis:

1. Why only $tRNA^{Trp}_{CCA}$ from *B. nonstop* is mentioned, although your paper in *Nature* demonstrates the effect of anticodon stem shortening also for $tRNA^{Trp}_{CCA}$ from the ciliate *Condylostoma magnum*? Is there any specific reason to be silent about this result, especially given the fact that it documents the generality of the principle?
2. Could you, please, elaborate on the wider implications of the fact that different eukaryotes have independently employed an alteration of the anticodon stem to secure a tRNA capable of decoding UGA as Trp? Why mutation of the anticodon itself has not been used? And what about eubacterial translation systems, including the organellar ones – would anticodon stem shortening work in them analogously to the situation in *Blastocrithidia* or *Condylostoma*?
3. Are the findings reported in the third paper (the manuscript in *Nature Structural & Molecular Biology*) applicable (at least in general) to prokaryotic translation systems, or is the role of interaction between ribosomal proteins and tRNA in securing the fidelity of translation unique for eukaryotes?

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