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Evaluation report of the thesis of <u>MSc. Terezia Prilepskaja</u> entitled "Analysis of mutual interactions of the human multifactor complex components in translation initiation" for PhD. award.

Dear chair, dear members of the committee,

it was a great pleasure to read the thesis of MSc. Terezia Prilepskaja. When I agreed to review it, my main motivations were as follows. First, I have known Terezia for quite some time (and now, thanks to my hazy memory, I realized that I also reviewed her bachelor thesis when she was still a virologist). My second, selfish reason was to keep my bearings in the field of eIF3 and translation initiation by reading the thesis, as the topic is broad and rapidly evolving.

Terezia's thesis is based on its full version and two attached publications. The first paper **"Structural Differences in Translation Initiation between Pathogenic Trypanosomatids and Their Mammalian Hosts"** was published in Cell Reports in 2020. As the paper is the result of an international collaboration, Terezia is one of the three first authors. **"Adapted formaldehyde gradient cross-linking protocol implicates human eIF3d and eIF3c, k and l subunits in 43S and 48S pre-initiation complex assembly, respectively"** is the second paper published in Nucleic Acid Research also in 2020, where Terezia is listed as second author. In both publications, she has made significant contributions by preparing dozens of constructs and performing in vitro binding studies. She has also contributed to the writing of articles and preparation of figures. Since both papers have been published in high IF journals, and more importantly, are respected by the scientific community, and finally have already been peer reviewed, I focused mainly on the PhD thesis itself.

As mentioned above, Terezia wrote the full version of the thesis, which consists of the chapters Introduction, Material and Methods, Results, Discussion and Conclusions. The Introduction provides a concise overview of current knowledge on translation initiation in eukaryotes and start codon selection. It also explains in detail the roles of translation initiation factors 1, 1A, 5 and 3, including the subunits of factor 3, in the above processes. Given the breadth of the topic, I appreciated that Terezia kept the focus on information that the reader should know before reading the "Results" chapter and carefully balanced depth of insight with the fluency of the text. I have no criticisms of the "Materials and Methods" chapter as it is a mandatory part of the thesis, and there is no reason to question, for example, why some chemicals, materials or enzymes are listed, and some are not. Rather, this chapter serves to give the reader an idea of the amount of work that Terezia had to do, which is well illustrated, for example, by the list of constructs used.

The main project presented in this thesis was to investigate the interaction of eIF3c and eIF5 and their function in maintaining start codon selection fidelity. It was shown that downregulation of eIF3c leads to the disassembly of the eIF3 holocomplex into the YLC and the f-h-m subcomplex, with concomitant loss of the e-d-k-l subunits. Downregulation of the c subunit increases the frequency of non-canonical initiation codon usage (especially UUG

and AAG), suggesting that eIF3c plays an important role in maintaining start codon fidelity (which was not observed for eIF3k and I knockdowns). In addition, depletion of eIF3c leads to upregulation of eIF5 at both mRNA and protein levels. Next, Terezia focused on defining the eIF5 binding site in the C-terminal domain of eIF3c. To this end, she performed a comprehensive mutagenesis and in vitro binding assays and mapped the binding site near the C-terminus of subunit c. Just to investigate the impact of loss of eIF3c and eIF5 interaction on start codon selection in vivo, stable HEK293T cell lines with R3N and F5A mutation in eIF3c gene, respectively, were generated and validated. A phenotype of decreased start codon selection fidelity like that of eIF3c downregulation was observed. I would like to mention here that I highly appreciate the efforts to use the CRISPR/Cas9 system to introduce mutations into the genome, as this approach is more complex than simply knocking out a gene. Terezia conducted cell line generation with all required controls, carefully checked all steps of whole procedure and, I guess, introduced CRISPR/Cas9 technique to laboratory also for use by others.

Having been very pleased with the Introduction and Results, I must highlight the Discussion, which is one of the best discussion chapters in theses I have read so far. This chapter demonstrates what I consider to be the preferred method of scientific work, i.e., studying the literature - building a model for testing - designing experiments - conducting experiments - interpreting the results and comparing them with the original model - refining the model. Terezia discussed results of published articles and those presented in the thesis together, compared them with literature and propose future work.

The thesis is written clearly and concisely, in good English; mistyping errors are rare, somehow more frequent in later parts of the thesis (but I do not mention them here because they do not hinder the understanding of the text or lead to confusion).

I have only minor comments/questions to thesis; they are listed below:

1. Page 27. Does the reduction in the number of eIF3 subunits to five in *S.c.* reflect their secondary loss during its evolution, or does the Saccharomycetales represent a separate clade?
2. Page 45. Is there any particular reason why you used 9% FBS instead of the conventional 10% in the HEK293T cultures?
3. Page 46. I would prefer to write the relative centrifugation force as "number x g".
4. Page 58 and others. There is inconsistent writing of initiation codon sequences in the text. I am referring to their notation in the DNA sequence instead of the RNA sequence, although plasmid reporters are not meant. On the other hand, I fully understand the reasons for writing them as DNA, which reflects the author's mind-set after constructing the vectors in question.
5. Figure 10. Downregulations of eIF3c, k and I are demonstrated by WB and qPCR, since no densitometric quantification of WBs is shown, I am confused as to whether the numbers of biological replicates marked above columns in Fig. 10A reflect different wells transfected with various reporters in one siRNA knockdown experiment or data are derived from several independent siRNA experiments? Luciferase activity was measured in HeLa cells, do you have any results for HEK293T (for direct comparison with CRISPR/Cas9 cell lines)? Do you have any explanation why the anti-eIF3I antibody detects two bands?
6. Figure 11. Downregulation of either 3c or 3d subunits causes an increase of eIF5, both in mRNA and protein level, but eIF5 protein is more upregulated and eIF5 mRNA is less upregulated in eIF3c knockdown in comparison to 3d knockdown, where the opposite is true. How do you explain this observation?
7. Figure 13. I understand that RNAseq and Ribo-seq data shown are rather illustrative just to support WB results, but still, I would suggest including some details of described experiment, such the number of biological replicates and P*adj* values, just to get idea how relevant are the ratios depicted in the heatmap. How was the statistical significancy in the heatmap calculated? How was TE calculated?
8. Figure 16. The legend seems to me a little bit confusing. It is an alignment of two genomic regions, blue box should depict 5´UTR of transcript variant 4 encoded by the eIF3c gene, but accession number refer to protein isoform, not to mRNA, and indeed, displayed 5´UTR sequence is not complete (NM_001267574.2). Yellow box denotes CDS, not exon 1 (which does form 5´UTR and overlaps with CDS). May be, I did not understand correctly, in any case, I feel it as not so much important because the alignment should demonstrate identical sequence in the surroundings of intended mutations.

9. Figure 22 - Legend. This is more about testing the homozygosity of the respective mutant allele in the clones rather than determining the ploidy of the clones.
10. Figure 23 - legend. I prefer marking pGL4 as pGL4-eIF3c-NTT (or similarly) because pGL4 to me is a backbone of the vector.
11. Figure 24D. ANOVA seems to me more suitable for the calculation of statistically significant variance than one-sample t-test.
12. Figure 25C. PCR efficiency calculation and melting curve analysis for the primer pair tested are a more convenient way to present optimization of primer concentration and annealing temperature (which, of course, assumes working with different cDNA dilutions).
13. Figure 26. Reduced binding between eIF5 and eIF3c results in reduced fidelity of start codon selection, however, did you test binding of mutant eIF3c variants to eIF5 in HEK293T in vivo?

Questions:

a) You mentioned in your Abstract that your ongoing project will be to generate a mouse model to study the function of eIF3. I found this goal interesting because it is probably time to move translation research to the level of whole organism in general. I would like to ask how the transgenic mouse model will be prepared. What phenotype would you expect to see in mice mutant for the eIF3c R3N and F5A alleles (non-viability, embryonic lethality, tissue, or organ specific phenotype)?

b) You were concerned about the lethal phenotype of eIF3c mutants in stable cell lines. Finally, F5A and R3N had only subtle effect on relaxation of start codon selection fidelity in HEK293T cells, which was even weaker than that observed after downregulation of eIF3c by siRNA-mediated knockdown. Have you thought about generation of HEK293T cell line with the R3NF4AF5A mutation?

c) Can you explain why R3NF4AF5A triple mutation does not abolish binding to TIF5 in yeast unlike in human?

d) Did somebody test point/deletion mutants of N-terminal domain of eIF2beta? What are their phenotypes (either observed or intended)?

Taken together, I rate this work very highly and I am convinced that the thesis and the attached publications of MSc. Terezia Prilepskaja meet not only the recognized standards of Charles University for the PhD award, but also international standards. Therefore, **I gladly recommend the acceptance of this PhD thesis** and wish Terezia a successful and meaningful professional career.

RNDr. Tomáš Mašek, PhD   Prague, May 24, 2024