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Evaluation of a Ph.D. thesis

Johana Kollárová: "Regulation of gene expression at posttranscriptional levels"

The thesis comprises two projects on which the candidate has participated over the period of her Ph.D. studies. The exact duration of this period is hard to know as neither the thesis itself nor its abridged version include the candidate's CV. Judging from the submission date in 2012 of the first paper that constitutes this thesis, the study period must have been at least seven years. There is one published paper by Mikolas et al 2013 in *PLoS One* in which the candidate is the second author. The other paper contributing to the thesis is the candidate's first-author manuscript *in press* in the Faculty's in-house journal *Folia Biologica*.

I will first comment on the organization of the thesis, then on the two featured papers. The thesis is introduced by an overview (p.14-32) which is far too broad as it attempts to capture gene regulatory mechanisms at all diverse transcriptional and some posttranscriptional levels. This inevitably leads to superficial skimming through those vast areas, with only some of the information being directly relevant to the thesis projects. I would argue that the attempt to unify the thesis topics under "posttranscriptional levels" generally fails, as at least the project on the transcriptional corepressor GEI-8 is entirely "transcriptional". Likewise the rationale given on p.13 is written in fuzzy language and lacks focus. It would have been better to title and organize the thesis based on the actual contents.

One negative aspect is that large sections of the thesis including part 1.3 of the Introduction, Materials and methods, Results and partly even Discussion (i.e. p.29-74) are essentially copy-pasted (with minor modifications of the text but identical figures) from the two presented papers, particularly from the *Folia Biologica* manuscript. Therefore the same material is printed in the thesis twice, the second time as "Supplementary files". Duplication is an effective way to add volume but it greatly reduced enthusiasm in this reviewer.

Given the 46 redundant pages, it is paradoxical that the author omitted Supplemental Information published with the *PLoS One* paper, which contains valuable information. The thesis also lacks two elements important for knowing the candidate: (1) a CV summarizing the candidate's activity over the project duration, and (2) explicit description of the candidate's actual contribution to the presented projects. This makes it hard for an external reviewer to judge the candidate's achievements and skills. On the positive note, the thesis is well presented and the English is of high standard (who wrote it?).

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The first of the two projects concerns the *C. elegans* ortholog of the transcriptional corepressors NCoR/SMRT, discovered in the mid-1990's as proteins recruiting histone deacetylases to genes bound by certain nuclear receptors in the absence of their ligands. The team including the candidate as a second author identified and partly functionally verified the product of the *gei-8* gene as a nematode NCoR/SMRT. Unfortunately the GST pull-down (Fig. S1) showing interaction of GEI-8 with the nuclear receptor NHR-60 is not part of the thesis. Using a *gei-8* allele generated by the *C. elegans* Knockout Consortium they described an array of *gei-8* mutant phenotypes from neuronal function, locomotion, pharyngeal pumping, body length, and gut malformation to defects in gonad morphogenesis. Such a pleiotropic impact is to be expected given the multitasking nature of these corepressors. In the Discussion (p.58) the candidate states that the *gei-8* deletion allele is likely null. This seems unlikely given the late arrest of the homozygotes and the premature stop codon introduced relatively late in the GEI-8 sequence. The published text also argues against possible dominant-negative effects of the protein truncation, which in my opinion has not been rigorously excluded. The authors further speculate that development in the *gei-8* homozygotes might be sustained by maternally provided *gei-8* products. Regarding these points I have two questions:

Q1: What genetic experiments are needed to ascertain a null nature of an allele and exclude its dominant-negative effect?

Q2: Working with *C. elegans*, what are the obvious experiments that should have been done to address potential maternal effects? And why were they not done?

Quite surprisingly for a putative repressor, many more genes were found downregulated in the *gei-8* mutants than genes that were upregulated (presumably derepressed). One plausible explanation (that GEI-8 represses another repressor) is offered on p.59 but immediately denied by absence of known repressors among the microarray hits.

Of 275 upregulated genes, the authors chose to study one encompassing both an enzymatic activity (*sqrd-2*) and 21U RNAs related to the piRNA pathway. RNAi targeting of this ensemble (locus Y9C9A.16) neither induced any visible defect by itself nor suppressed the phenotype of the *gei-8* mutants (which one might expect as loss of GEI-8 leads to *sqrd-2* overexpression). Instead, knockdown of either *sqrd-2* or its paralog *sqrd-1* led to novel defects in gonad formation. The authors interpret this as a genetic link between *gei-8* and *sqrd-2/sqrd-1*, and speculate about potential involvement of 21U RNAs as well. I am not convinced that either is supported by their data. It just seems that the *gei-8* mutants provide a sensitive background on which loss of *sqrd* (or any other genes?) becomes critical.

Further, according to the Discussion (p.59 in thesis and p.11 in Mikolas et al, 2013), *sqrd-2* RNAi applied in the *gei-8* homozygous background caused a "partial reversal of the *gei-8* mutant phenotype". However, this is contradicted in the Results (p.10 in Mikolas et al, 2013): "We predicted that knockdown of the expression from Y9C9A.16 locus in *gei-8 (ok1671)* homozygous mutants might revert or modify some of the observed phenotypes; the latter was observed." Which statement is true?

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Indeed I could find no data for phenotype suppression in Mikolas et al (2013) or in the thesis. The candidate should clarify the above issues:

Q3: What is the evidence for *gei-8* phenotype suppression by *sqrd-2* knockdown and for a specific genetic interaction between the two genes?

Q4: Which experiments indicate involvement of 21U RNAs (as opposed to the SQRD enzymes themselves) in the observed gonadal phenotypes?

In summary, the first paper presented in the thesis provided important primary characterization of the NCOR/SMRT ortholog in *C. elegans* although it has not elucidated its mechanism of action. Since its publication in 2013 the work has had a moderate impact.

The second paper explores a gene encoding an enzyme ALKB-8 possessing RNA-binding and methyl transferase activities with implications in translational control at the level of tRNA modification. The authors observed a widespread expression pattern throughout the worm life cycle. Surprisingly *alkb-8* RNAi worms displayed accelerated reproductive maturation rather than any visible developmental defect. Loss of *alkb-8* led to increased Nile red staining in the gut, whereas heat-shock driven overexpression of *alkb-8* let to lower Nile red staining. However, in my opinion these data are incomplete and inconclusive without necessary controls (see Question 7). The authors claim that while *alkb-8* RNAi had no effect on lifespan whereas *alkb-8* overexpression extended it. However, also this experiment is not properly controlled (see Question 7).

Q5: The section (l.403 on) "The effects of *alkb-8* downregulation and forced overexpression on *C. elegans*" says nothing about *alkb-8* overexpression. The next section starts about "involvement of ALKB-8 in the function of lysosome-related organelles" without any previous connection or rationale for doing the Nile red staining. Something must be fundamentally wrong here. A part of the data missing? Please clarify.

Q6: Is the 50-fold increase in egg number laid by *alkb-8* RNAi worms strictly given by the early onset of oogenesis or do the *alkb-8* RNAi hermaphrodites also produce more eggs over the entire reproductive phase? Was the total egg production determined? Both accelerated development and enhanced fertility would be expected to shorten lifespan of *alkb-8* RNAi worms, which is not the case considering Fig. 8A. Any explanation?

Q7: Where are the controls for Fig. 7? For a heat-shock driven gain-of-function experiment, one needs two types of controls: a wild-type reference line both with and without heat shock, treated in parallel to the transgenic line(s), again with and without heat shock. However, Fig. 7 does not assess the effect of heat induction of ALKB-8 as it lacks no-heat shock control of the second type, and the "control" fails to indicate effect of the heat shock alone, without ALKB-8 induction. Thus the experiment depicted in Fig. 7 lacks half of the data and is effectively meaningless. Because data are not being compared between controls, *alkb-8* overexpression, and RNAi, it is also impossible to tell if gain of ALKB-8 reduced Nile red staining just to the levels seen in wild-type worms or below normal. Is that known?

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Similar to Fig. 7, also Fig. 8B fails to indicate genotype and treatment of the control group and fails to address effect of heat-shock alone versus specific effect of ectopic ALKB-8. Whether the longevity extension was statistically significant is also not assessed. I expect the candidate to critically address these apparent shortcomings during her defense.

Overall, in my opinion this study is a collection of preliminary and mutually disconnected data without clear links between the suspected *alkb-8* gene function, expression, and the altered Nile red pattern. A functional link to metabolism is missing and the rationale for assessing longevity is unclear. Unfortunately, the data on Nile red staining and lifespan extension are inconclusive due to the lack of critical controls.

Summary. The thesis presents a large body of modern methods in molecular genetics and thus it was an opportunity for the candidate to learn many approaches instrumental in current research. The problem is that without knowing which of the experiments were designed, performed, and interpreted by the candidate, it is difficult to judge which skills exactly she has attained. From the "Goals" vaguely described on p.13 I am guessing that the candidate was responsible for the microarray analysis of genes downstream of GEI-8 and for most of the work on the ALKB-8 project, but this should be clarified at the defense.

Because the Czech system obligates reviewers to assess a thesis before defense takes place, it is difficult to explicitly answer the question 'whether the thesis demonstrates the author's ability to conduct independent scientific research' as requested by the protocol. This would normally depend on the how the candidate performs at the defense. Therefore, I will assume that the first and second positions on the presented papers automatically imply major contributions of the candidate and hence justify successful completion of the graduate studies. In conclusion, I herewith recommend that based on her thesis entitled "Regulation of gene expression at posttranscriptional levels", Johana Kollárová be granted the Ph.D. title.

Sincerely,

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