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Review of a Ph.D. dissertation of Mr. Kishor Gawade, M.Sc., entitled "FUS-dependent processing of snoRNAs into sdRNAs and regulation of ribosomal RNA modifications: implications in Amyotrophic Lateral Sclerosis (ALS)"

The dissertation subject and its scientific significance

The subject of Mr. Kishor Gawade's dissertation was to describe the landscape of snoRNA processing and the associated post-transcriptional changes in rRNA depending on the presence of the correct FUS protein. Mutations in the gene encoding this protein lead to ALS, a fatal neurodegenerative disease. Similarly, mutations in other genes, such as *TDP-43*, are also likely to lead to changes in RNA metabolism that result in ALS. This suggests that inadequate control of this biological process is crucial for the observed neuronal death in ALS. Therefore, a comprehensive description of the changes in the expression of different RNA classes and their post-transcriptional modifications in ALS at different stages of neuronal development is fundamental, both for understanding the molecular pathogenesis of ALS and, more generally, the role of RNA metabolism in neuronal development, function and death. The work submitted to me for review addresses these questions in detail, using advanced cellular models and next-generation sequencing methods. Although it only provides correlation evidence and not cause-effect relationships between the changes in the metabolism of the RNA studied and the development of ALS, it represents an important step towards finding the latter in the future. Therefore, I believe that the work submitted to me for evaluation should be considered innovative and potentially significant for the advancement of knowledge.

Formal description of the Ph.D. dissertation

The dissertation has a rather unusual structure, as it is a mixture of appended published work and a description of unpublished results. I want to emphasize that, in my opinion, this is not a significant problem. The dissertation consists of 72 pages and two articles published in international journals. It is preceded by a list of the doctoral student's publications (1 page, 3 items), a list of abbreviations used (3 pages), information on the source of funding for the research described (1 page), summaries in Polish and English (4 pages in total), background and forming hypothesis (2 pages in total), introduction (4 pages), summaries of the content of the attached published papers (2 chapters, 4 pages in total), description of unpublished results (2 separate subchapters with sections on materials and methods, results and discussion; 33 pages in total), references (4 pages, 68 points) and statements by the authors and co-authors (12 pages). The attached published manuscripts are the experimental paper by Gawade *et al.* (Scientific Reports, 2023) and the review article by Gawade and Raczynska (WIREs RNA,

2023). It is clear from the authors' statements that Mr. Gawade played a leading role in formulating hypotheses, planning, and performing experiments, and preparing the manuscripts. However, they refer to the description in the "Authors' contribution" section of the articles, which are not very detailed. Therefore, it is difficult to assess exactly which experiments were performed by Mr. Gawade and which were performed by collaborators. I believe that the author's and/or co-authors' disclosures could be somewhat more detailed to clarify this matter. As part of my formal duties as a reviewer, I note that, apart from the published results section, the submitted paper contains 15 figures and 6 tables.

Evaluation of the merits of the dissertation

Due to the structure of the dissertation, it is difficult to refer to the traditional elements of the dissertation. Therefore, I will focus mainly on those parts of the dissertation that contain materials and methods as well as results and their discussion, as I have no major comments on the summaries, the general introduction and the stated hypothesis and objectives of the dissertation. They are clear and informative. In my opinion, the hypothesis and objectives are formulated correctly although the latter ones could be less diverse. My only comment on the general parts is that I missed an additional subchapter "General Discussion" where the totality of the results obtained would be discussed synthetically. This is a practice used in many countries where dissertations consisting of published articles are allowed.

Publication 1: Gawade et al., 2023, Scientific Reports 13: 2974.

Three fundamental questions were posed in this part of the dissertation:

(i) Do wild-type cells and cells lacking FUS expression differ in the expression patterns of snoRNAs?

(ii) Does the differentiation status of the cells influence the expression of snoRNAs?

(iii) Do these differentially expressed snoRNAs lead to ribosomal RNA modifications such as 2’-O-ribose methylation and pseudouridylation?

Therefore, the main aim of this work was to understand whether changes in snoRNA expression in response to FUS status could lead to a different ribosome pool in healthy cells compared to cell models of ALS. Since ALS is largely considered a disease of mature neurons, experiments were performed not only on the standard non-neuronal cell line HEK293, but also on SH-SY5Y cells that differentiate into neuron-like cells under appropriate culture conditions. Both wild-type and FUS-deficient cell lines were used for the basic analyzes. In the case of SH-SY5Y cells, a cell line carrying a mutated copy of the FUS gene, which correlates with the occurrence of an aggressive form of ALS, was also included in the analysis. Modern next-generation sequencing methods were used to analyze the expression of small RNAs and post-transcriptional changes. Particularly noteworthy is the use of unique methods such as RiboMeth-seq and HydraPsiSeq.

As a result of the research, significant differences in the expression profiles of snoRNAs were detected in the cells studied, depending on the cell type, their degree of differentiation and the presence or absence of functional FUS. Furthermore, a significantly more intensive post-transcriptional modification of rRNA was observed. Additional bioinformatic analyses showed that the changes in snoRNA expression levels correlate, at least partially, with the observed post-transcriptional changes in rRNA. A potentially very important consequence of these changes would be the difference in ribosome heterogeneity available in the cell, which could influence translation efficiency. However, an examination of the global translation level did not reveal any fundamental differences in the efficiency of protein synthesis.

In summary, the results obtained have significantly increased our knowledge of the changes in RNA metabolism both during cell differentiation and potentially in the absence of FUS, leading clinically to ALS. In my opinion, a weaker aspect of this work is the use of, at most, neuro-like models to analyze disease-related processes specific to mature neurons. However, this aspect has been addressed in the section where unpublished results are presented. The second weaker point is the lack of attempts to establish a cause-effect relationship between the observed molecular changes and disease processes. However, it must be honestly stated that this may not be possible at this stage of research progress. Finally, it seems that the application of proteomics and analyses focusing more individually on differential synthesis of specific proteins would show how changes in ribosome heterogeneity in the cell translate into potential changes in translation levels in ALS.

Regarding more specific comments, it is unclear why a t-test was used for Figures S7 and S9. On the one hand, the control variant equals 1 and has no variation. On the other hand, in some cases (e.g., EWSR1 for SH-SY5Y Diff), there is more than one group compared to the control variant. In my opinion, a more appropriate test would be the one-sample t-test.

Publication 2: Gawade and Raczynska, 2023, WIREs RNA: e1818

The second published manuscript of the dissertation is a review article addressing the potential role of two loci encoding snoRNAs that are subject to imprinting in development and genetic disorders. It is undoubtedly a comprehensive analysis and a valuable piece of work. However, I am not convinced that this manuscript should be included in the doctoral thesis, as, in my opinion, it does not constitute a directly original solution to the scientific problem, which should be one of the fundamental characteristics of a doctoral thesis according to the relevant regulations.

Unpublished results 1. FUS mutation dependent changes in ribosomal RNA modifications in ALS patient-derived fibroblasts, iPSCs, smNPCs, and motor neurons.

This section of the dissertation continues the research presented in the paper mentioned above by Gawade *et al*. (2023). However, in this case, alterations in 2'-O-ribose methylation of rRNA were compared in fibroblasts from ALS patients (different mutations causing different clinical symptoms, from moderate to severe ones) and in iPS cells and their derivatives (e.g., smNPCs and motor neurons). In the first case, the focus was on the comparison of patient cells with correspondingly matched control cells. In the case of iPSCs and their derivatives, the FUS P252L mutant cells and isogenic controls were examined. As a result of the analyses performed, it was found that the level of rRNA modification examined increases with cell differentiation. Consequently, the most significant changes in 2'-O-ribose methylation between healthy and diseased individuals were observed in fibroblasts and iPS cells and, to a lesser extent, in smNPCs and motor neurons. These observations are, in my opinion, fascinating, not only in the context of ALS, but also in general disease modeling with iPS cells and their derivatives, as they suggest that certain molecular differences may decrease with age, or, as the authors claim, that this method of modeling diseases of mature neurons may not reflect potential real differences because of the reprogramming process from patient-derived cells to pluripotent cells.

However, I have some critical comments on this section. In my opinion, the presentation of the results is less polished in terms of editorial quality compared to the published data. This sometimes makes it difficult to understand the methods or data presented. For example, the methodological description of the differentiation of the cells studied is split between Fig. 2 and Table 2, leaving the reader to calculate the final concentrations of the trophic factors used.

There is also no description of how RiboMeth was performed, and the source of various key materials and catalog numbers is missing. In addition, Table 3, which lists the fibroblast lines as material, has ended up in the results section.

As far as the presentation of the data is concerned, it is not entirely stringent. For example, the meaning of the different colors used in the PCA analysis of the individual cell types is not explained in Figure 10. Moreover, it would be advisable to present the PCA analysis separately for each cell class to better highlight possible differences between control cells and ALS models. Furthermore, the description of Fig. 3 is somewhat chaotic and scale bars are missing. In the results presented in this figure, the Ph.D. student claims that mislocalization of FUS is visible in certain lines but not in others. However, I missed a quantitative analysis of these observations. I also wonder why the analysis of FUS distribution was only performed for fibroblasts and not for iPSC cells, smNPCs or motor neurons. Was this effect also observed there? If not, could its absence be an alternative explanation for the minor differences observed in sequencing between healthy and "diseased" cells?

Another example of poor data representation is Fig. 7, where the images are too small to make meaningful comparisons. However, one thing that could be observed is the very low number of β-TubIII- and MAP2-expressing neurons in the FUS P252L variant. There is literally only one cell positive for MAP2, and the staining is not in the dendrites where MAP2 should be present. Nevertheless, the Ph.D. student states that the cells have successfully differentiated into motor neurons. It is not clear to me on what basis this is claimed. Perhaps non-representative images were used.

Unpublished results 2. FUS-dependent processing of snoRNAs into sno-derrived RNAs (sdRNAs).

The main question in this part of the thesis was whether snoRNAs are processed into sdRNAs depending on the differentiation stage and FUS. To clarify this, an additional bioinformatic analysis of the data from small RNA sequencing of SH-SY5Y cells was performed in the study by Gawade *et al*. 2023. As a result, the Ph.D. student concluded that there are fundamental differences in snoRNA processing between proliferating and differentiated cells. In addition, differences were found for certain snoRNAs depending on FUS. These are exciting observations; however, their physiological significance and relevance for the development of ALS were not the subject of this work. Undoubtedly, this will be the subject of further studies in Prof. Raczynska's laboratory.

Final conclusion

I, hereby, declare that the reviewed Ph.D. thesis by Kishor Gawade meets the conditions specified in Article 187 of the Act of July 20, 2018. Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended), and I request the Council of the Discipline of Biological Sciences of the Adam Mickiewicz University in Poznań to admit Kishor Gawade to the further stages of the proceedings for the awarding of the degree of Doctor of Science in the field of science in the discipline of biological sciences. Furthermore, considering the substantial scientific value of the presented data and the necessity for the doctoral candidate to employ modern research models, methods, and bioinformatic analyses, I recommend awarding Mr. Kishor Gawade's doctoral dissertation with distinction.

Jacek Jaworski