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Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Evgeniia Shcherbinina

PhD Program: Life Sciences

Title of Thesis: Role of lncRNA LL35 in hepatocytes function

Supervisor: Dr. Timofei Zatsepin, Velocity Global Rus

Name of the Reviewer:

I confirm the absence of any conflict of interest (Alternatively, Reviewer can formulate a possible conflict)	**Signature:** **[Richard Lathe]** **Date: 11-August-2022**

The purpose of this report is to obtain an independent review from the members of PhD defense Jury before the thesis defense. The members of PhD defense Jury are asked to submit signed copy of the report at least 30 days prior the thesis defense. The Reviewers are asked to bring a copy of the completed report to the thesis defense and to discuss the contents of each report with each other before the thesis defense.

If the reviewers have any queries about the thesis which they wish to raise in advance, please contact the Chair of the Jury.

Reviewer's Report

Reviewers report should contain the following items:

- Brief evaluation of the thesis quality and overall structure of the dissertation.
- The relevance of the topic of dissertation work to its actual content
- The relevance of the methods used in the dissertation
- The scientific significance of the results obtained and their compliance with the international level and current state of the art
- The relevance of the obtained results to applications (if applicable)
- The quality of publications

The summary of issues to be addressed before/during the thesis defense

Basis of evaluation

A research thesis is expected to be an original piece of empirical work of relevance to biomedical science, demonstrating the candidate's ability to apply scientific principles and undertake rigorous investigation. It should be of publishable quality, make a distinct contribution to the knowledge of the subject, and afford evidence of originality.

Field of research: role of long non-coding (lnc) RNA LL35 in cancer and the cell cycle

The thesis focuses on understanding the biology of mouse lncRNA LL35, that is inferred to be the functional homolog of human DEANR1.

In human, DEANR1 has been reported to be a tumor-suppressor protein that binds to transcription factor GATA6, as well as decreasing cancer cell proliferation and metastasis through epigenetic regulation of c-Myc. Moreover, other teams reported that human DEANR1 directly binds to FOXP3 (a transcriptional regulator) and modulates oncogenesis in several cancer models. It also acts by binding small RNAs.

The thesis proposes that LL35 is the equivalent to DEANR1 because (i) both DEANR1 and LL35 are located downstream of the FOXA2 gene; (ii) both DEANR1 and LL35 appear to positively regulate *FOXA2*/*Foxa2* gene expression; (iii) the promoter region and first exon of LL35 are well conserved between mice and human, as well as in other mammals, but the rest of the lncRNA sequence is poorly conserved between DEANR1 and LL35, raising the question of whether LL35 and DEANR1 truly have similar biological roles.

This is a key issue addressed by the thesis.

Theoretical background

The introduction to the thesis comprehensively reviews our understanding of the roles of lncRNAs in diverse aspects of biology. The candidate describes and references multiple mechanisms, including direct lncRNA binding to cellular targets including both RNAs and proteins, leading to modulation of histone modifications, transcription regulation, mRNA splicing, stability, and translation, the binding of other small RNAs, and in this way can govern multiple aspects of cellular metabolism. In addition, the thesis correctly points out that some lncRNAs in fact encode short peptides that themselves have regulatory activities.

Data presentation

The thesis is presented in a clear and logical order. The figures are well-designed, and the methods section comprehensively describes the diverse cellular and molecular methods. In addition, the candidate has contributed to four key papers in peer-reviewed journals.

Technical training

Scientists increasingly work together as part of a team, and add their skills to those of their colleagues to generate a team whose joint output could not be achieved by a single individual working alone. The candidate properly states in the thesis which techniques were performed by herself and which relied on other members of the team.

The basic experimental system used by the candidate and the host laboratory involves mouse hepatocytes, expansion and differentiation *in vitro*, and *in vitro* and *in vivo* delivery of antisense oligonucleotides as an analytical approach. The thesis presents an impressive list of diverse molecular and cellular techniques, and these have been successfully employed by the candidate to interrogate the function of LL35. There is no doubt that the candidate is familiar with diverse methodologies that are of great utility in the biosciences.

In support of that conclusion, the candidate is coauthor of three papers, and lead author of a fourth. These are summarized below.

Belicova *et al.* (2021) is published in a prestigeous journal (*J. Cell Biol.*). The paper describes using knockdown approaches how Rab35 plays a role in the switch from bile duct-like (conventional apicobasal) polarity to biaxial complex polarity.

Sergeeva *et al.* (2021) in *Int. J. Mol. Sci.* investigate the role of DDX3 in cell-cycle regulation. Again using knockdown, the authors report that depletion of DDX3 leads to reduced cell proliferation and increased apoptosis.

Kovalenko *et al.* (2022) in *Biochimie*, using similar techniques, explore how linc-RoR modulates glioblastoma differentiation.

The fourth paper, Shcherbinina *et al.* (2022) in *Biomedicines*, is the most important in the context of this thesis because it relates directly to the doctoral thesis under examination – and describes experiments designed to determine the role of LL35. This paper and the thesis itself are evaluated together below.

Key experimental results

1. The key observation is that LL35 is downregulated in mouse liver in fibrosis/proliferation, including CCL4 treatment, hepatocellular carcinoma, and partial hepatectomy, arguing that LL35 is, like DEANR1, is a tumor-suppressor.

2. However, in further experiments it was found that LL35 knockdown had few to no obvious phenotypic effects *in vivo*. It was confirmed that knockdown reduced LL35 levels by 81–84%.

3. To explore this further gene expression profiles were investigated after knockdown in AML12 cells *in vitro* and in liver *in vivo*. 796 changes were detected (523 upregulated, 273 downregulated). Only 5 changes (0.5%) were common to AML12 and liver.

4. Proteomic analysis following knockdown using PANTHER Pathway analysis revealed that cell-cycle functions, rRNA processing, and lipid metabolism were altered in AML12 cells, whereas cell-cycle functions were unchanged in liver. There are some conflicts, for example Chek1 was upregulated by knockdown in AML12 cells but downregulated in liver.

5. Further proteomic analysis using LC-MS found 232 changes.

6. Lipidomics using LC-MS. Also many changes, but few changes common between AML12 and liver.

7. Metabolomics. Changes in purine metabolism were documented.

8. Glucose metabolism. LL35 knockdown causes upregulation of G6PC and PEPCK, which to some extent

is the reverse of what one would expect for a tumor-suppressor function. However, the changes were small.

9. Insulin tolerance in liver knockdown mice. Blood glucose was 1.5–2-fold increased in insulin-treated knockdown mice versus insulin-treated controls. There appeared to be no changes in resting glucose.

10. LL35 knockdown and cell behavior *in vitro*. Knockdown was found to impair cell migration and proliferation, and leads to cell-cycle arrest in S-phase. This would appear to contradict the 'tumor-suppressor' function of LL35, because knockdown would be expected to increase cell proliferation (result #1). Further experiments looked at Jagged, Notch, and NF-kB signaling, but no striking new results emerged.

Critique

Using a single experimental strategy based on LL35 knockdown, the candidate has investigated multiple parameters that might be affected using an impressive array of analytical techniques. The broad outcome of this study is as follows:
(i) Small changes were seen in almost all parameters investigated, from gene expression, to pathway analysis, to proteomics and metabolomics and lipidomics, to glucose metabolism and insulin tolerance, to cell division.
(ii) The statistical analysis was purely by pairwise t-testing.
(ii) Results from *in vivo* and *in vitro* knockdown were different.
(iii) In some cases the results obtained argue against the contention that LL35 is a tumor-suppressor.

The overall picture is that none of the results identify a major role of LL35, and none of the effects of knockdown were remarkable. It is therefore unclear whether the central conclusion of the thesis – that murine lncRNA LL35 is the functional analog of human lncRNA DEANR1 – is substantiated by the data.

Overall assessment

The thesis is well written and provides ample evidence that the candidate has acquired many important skills in biomolecular technology. It is unfortunate that major functions and phenotypes were not uncovered despite diligent experimentation. This does not detract from the thesis – few doctoral theses deliver stunning new results that change the face of biology. For these reasons I recommend that the thesis may fulfill the requirements for the award of a doctoral degree.

Suggested revisions and amendments

1. The grammar of the title should be amended to read: Role of lncRNA LL35 in hepatocyte function (singular case for hepatocyte).

2. The introduction should be slightly expanded to include an additional (or supplementary) figure addressing sequence similarity between LL35, DEANR1, and corresponding sequences in other species. It is important to answer the question of how conserved the lncRNA sequence is, and whether similar sequences are present (or not) in other related metazoan species including marsupials, birds, amphibians, and fish, and possibly even insects, and to look at the divergence of LL35/DEANR1 sequences versus control sequences (e.g., FOXA2, other lncRNAs).

3. The conclusion of the thesis (LL35 is the functional analog of DEANR1) should be rephrased to reflect the uncertainties encountered in this work. The candidate should also give thought to what experiments

might be required to more accurately evaluate the relationship between LL25 and DEANR1.

These changes do not involve resubmission, but hopefully can be incorporated into the final accepted version.

Professor Richard Lathe
University of Edinburgh
August 2022

Provisional Recommendation

☒ *I recommend that the candidate should defend the thesis by means of a formal thesis defense.*

☐ *I recommend that the candidate should defend the thesis by means of a formal thesis defense only after appropriate changes would be introduced in candidate's thesis according to the recommendations of the present report*

☐ *The thesis is not acceptable and I recommend that the candidate be exempt from the formal thesis defense*