Skoltech
Skolkovo Institute of Science and Technology

Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Dominique Leboeuf

PhD Program: Life Sciences

Title of Thesis: UBR-ubiquitin ligases of the ARG/N-degron pathway as new targets for therapy: implications in cancer and inflammation

Supervisor: Associate Prof. Timofei Zatsepin

Name of the Reviewer: Dr. Emmanuelle Graciet

I confirm the absence of any conflict of interest	**Signature:**  **Date: 30/07/2020**

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

- **Brief evaluation of the thesis quality and overall structure of the dissertation.**

Ms. Leboeuf has submitted a PhD of extremely high quality, which presents potential novel approaches to treat hepatocellular carcinoma (HCC) using a combination of siRNAs targeting UBR-type E3 ligases of the ubiquitin-dependent N-degron pathway and known anti-cancer drugs. This PhD work has also uncovered novel roles for the N-degron pathway and new substrates that could play a role in the regulation of apoptosis and/or inflammation. The questions addressed and aims of the PhD are clearly outlined. The experimental and bioinformatic work was conducted using state-of-the-art approaches and careful design. The data is of high quality and the results have been thoughtfully analyzed and discussed, including relevant statistical analyses.

The PhD thesis is clearly structured, with each of the chapters forming a clear unit. Each chapter and their subsections are also linked to the original aims and research questions of the PhD work.

Ms. Leboeuf's thesis is clearly written in a concise style that is both easy and enjoyable to read, while bringing forward the complexity of some of the molecular mechanisms at play.

- **The relevance of the topic of dissertation work to its actual content**

In her PhD thesis, Ms. Leboeuf has addressed an important question relating to the improvement of cancer therapies, in the context of HCC. The work is well introduced in that Chapters 1 and 2 provide all the information needed to understand and explain the relevance of the research questions addressed during the PhD, as well as the methods used (especially siRNAs and lipid nanoparticles (LNPs)). The experimental work that is presented in subsequent chapters is directly linked to the introduction and the research questions outlined at the beginning. The concordance among the different sections and chapters forms a body of work that is highly relevant and logical.

- **The relevance of the methods used in the dissertation**

The experimental methods used by Ms. Leboeuf in her PhD thesis are state-of-the-art, in that they have been recently developed and/or have been shown by the scientific community to have a great potential to be used for cancer treatment. The combination of LNPs with siRNAs is a potentially viable option to treat cancer and other diseases. Other methods used to assess the effect of the siRNAs are also all modern and appropriate in the fields of cancer research, inflammation, immunology, the ubiquitin system and the N-degron pathway. In addition, as indicated clearly in the thesis, each method used has been carefully thought out, with pros and cons being outlined in the thesis, as well as the rationale for choosing specific methods.

The last chapter in Ms. Leboeuf's thesis relies on the combination of bioinformatics methods and transcriptomics to identify potential new substrates of the N-degron pathway that could play a role as new mediators of apoptosis. This is a highly relevant chapter and the approaches used yielded interesting predictions, that can serve for future work and applications.

The combination of both experimental research and bioinformatics approaches increases the scope of this PhD thesis. Similarly, Ms. Leboeuf has nicely combined the use of cell culture and animal models to first test reagents and obtain a proof of concepts for her different hypotheses, and then further validating into animal models.

- **The scientific significance of the results obtained and their compliance with the international level and current state of the art**

The main results presented in Ms. leboeuf's PhD thesis include:

(i) the identification of siRNAs and of a delivery approach *in vivo* that allow the efficient down-regulation of UBR-type E3 ligases known to degrade N-degron substrates in the liver. This not only served as the foundation to explore potential applications, but also allowed Ms. Leboeuf to uncover new roles of the N-degron pathway.

(ii) the discovery that a combination of doxorubicin, a widely used anti-cancer drug in the context of HCC, can be combined with a down-regulation of UBR-type E3 ligases to target and eliminate more efficiently cancer cells in HCC.

(iii) the discovery that the N-degron pathway plays a role in regulating inflammation. This is a significant advance that goes beyond the study of the role of the N-degron pathway in each of these cellular programs individually.

(iv) the identification of novel *bona fide* substrates of the N-degron pathway, and the discovery of new putative substrates with roles in apoptosis and/or inflammation. This aspect is highly relevant, because identification of N-degron pathway substrates has been notoriously difficult since the discovery of this ubiquitin-dependent protein degradation pathway in the mid 1980s. Ms. Leboeuf has used bioinformatics approaches that allowed her to make interesting predictions.

All of these results are substantiated by solid experimental evidence and clear explanation and analysis of the results. Ms. Leboeuf has also made an effort to present the statistical relevance (or not) of her results and has discussed this aspect in her thesis. I have found this to be of high standard, although some information needs to be added (e.g. number of replicates and n) for each experiment. This is highlighted below. The conclusions drawn are usually carefully presented and discussed.

- **The relevance of the obtained results to applications (if applicable)**

The results obtained by Ms. Leboeuf have a strong potential for applications to improve the efficiency of treatment for HCC patients, while also potentially decreasing the negative side-effects of chemotherapy and currently used treatments. Beyond the quality of the science and the results obtained, the potential for applications is best highlighted by the Russian patent application Ms. Leboeuf has filed (together with collaborators and mentors) on "*Composition and methods for downregulation of the Arg/N-degron pathway*".

- **The quality of publications**

In the course of her PhD work, Ms. Leboeuf has published 3 first-author papers, all of which have been accepted in well-known and accepted journals following a peer review process. I have found all 3 publications to be a significant advance the fields of research relevant to Ms. Leboeuf's PhD project, and also of high quality from a technical point of view.

Summary of issues to be addressed before/during the thesis defense

The PhD thesis submitted by Ms. Leboeuf is of outstanding quality. There are nevertheless a few minor points that would be ideally addressed before or during the defense.

Comments that apply to all sections:

In all results sections, an effort was made to present the results of statistical tests, however, the number 'n' of independent replicates or the number of animals/cells used are rarely specified. It would be very important to add this information to all figure legends.

Chapter 1:

- p2: very briefly indicate how sorafenib and regorafenib function. Why are they of relevance in this paragraph?

- p4: "ablation of the Arg/N-degron pathway in adult tissue, which has never been done before" - maybe consider rewriting this sentence to take into account Brower & Varshavsky (PLoS One. 2009 13;4(11):e7757).

Chapter 2:

-p6: section on E2 enzymes. The role of E2s in determining which of the 7 Lys residues of Ub are used for chain formation depends on the type of E3 (RING or HECT). This section should be nuanced more to present the situation more accurately. This can be done briefly.

- p8: first sentence to include bibliographic reference to Bachmair's 1986 paper when mentioning the author's names.

- p8: deubiquitylases are mentioned in the context of N-degron reporters. Maybe consider introducing them in the previous paragraph for clarity.

- p8: "N-degrons comprise: (1) a destabilizing residue" include N-terminal to improve clarity.

- Figure 2: This is a very good and comprehensive figure, but many details are not presented either in the figure legend or in the text when the different branches are detailed. Maybe consider adding a few sentences more in the text, particularly true for the fMet and Pro/N-degron pathways, or indicate in the figure legend what all the proteins indicated do? Should not be too long.

- p11: add Cys to the list of "tertiary" destabilizing residues?

- p13: Figure X??

- p17: (Ling) - incomplete reference

- p24: Dicer paragraph. When mentioning Arabidopsis for the first time, indicate that this is a model plant. It may be confusing for non-experienced readers, as you mostly talk about animal and yeast models in the thesis. I think it's excellent though that an effort was made to go beyond animals in the introduction and in the discussion.

- p35: BOX1 is extremely useful. It would be good to introduce it in the text with some details.

Chapter 4:

- p62: Asp-BRCA1 starts with aspartic acid, not asparagine.

- Figure 17b: correct legend of y axis. This is quantitation of protein, not mRNA, so “expression” may not be the best term.

- Figure 17 and other figures/experiments downstream. It seems that one or 2 different sets of siRNAs targeting the UBR E3 ligases have been used (e.g. figure 18; p63 etc…). What is the difference between these 2 sets: mixture of all siRNAs? identity of siRNAs? relative concentration? It is unclear if the results in Figure 17 were obtained with a set of siRNAs. This needs clarification not only in figure 17, but in general in the results section. Also, the identity of the siRNAs used in a given set should be specified. A table may be useful to do so concisely.

- bottom of p62 and Figure 18: results in figure 18 suggest that while there might be an effect of siUbrs on cell migration, it is not statistically significant. The conclusion at the bottom of p62 should be revised to reflect the data.

- Figure 20: unclear if a set of siRNAs or individual siRNAs were used. Review all figures and clarify.

- p67: please explain ALT, AST and ALP, including physiological relevance.

- Figure 27: the effect of LNP-siUbrs in HCC model is presented, with increased neutrophils and Ly6C^{High} cells. However, how does this compare to the LNP siUbrs in a normal liver? Are the effects specific to the HCC model? No additional experiments are requested, but if there is some data to discuss this, it would be good to include. If there is no data, then maybe still worth discussing?

- Table 7: why are proteins from hICAL downwards in a separate section of the table. Include an explanation?

- Figure 30 b/d/f & Figure 31: include error bars to half-life measurements? Effects are mild in some cases, so would be nice to have an idea of variation or error. Also specify number of replicates or cells if possible.

- Figure 34: no mention of LPS+ATP conditions. Why is that important and meaning of results - please comment.

- p100: origin of equation for prevalence value is unclear. Can this be specified? For example, is it derived from a previous publication? Is it based on empirical observations or theoretical calculations? If this equation is novel and part of the PhD work, then more details would be welcome to justify the terms and coefficients.

- Table 10: unclear if potential substrates in this table are putative or experimentally validated. Please clarify.

- p107: “downregulation of only one UBR Ub ligase will lead to a clearer picture…”. Please explain this argument/reasoning more clearly.

- p107: the number of differentially expressed genes (DEGs) is extremely small after removing background from siCtrl. Please comment on this in the text. I think you should also indicate the number of DEGs in each of the datasets before and after filtering background. Also including some Venn diagrams to show overlap would be good.

- p113-114: discussion of Agarwana and Banerjee 2016. Could the differences observed with results obtained in PhD thesis also be linked to different modes of action of shikonin and doxorubicin? Might be worthwhile discussing in more detail mode of action of these 2 drugs?

- p114: mention of expression pattern of UBR genes. Additional details and/or a figure might be beneficial. Not absolutely necessary.

<u>Formatting issues to address:</u>

Choose between commas and dots for decimals, but then be consistent with chosen notation. Also sometimes an apostrophe has been used to separate thousands instead of comma, but this is related to choosing a notation for decimals.

Some typos left.

Some mentions of N-end rule pathway instead of N-degron pathway.

Some abbreviations are not defined the first time they are used but later in the text.

“micro” indicated with a u instead of Greek letter for mu.

Some references in the text have a strange format. For example, at the top of page 1: (Collaborators 2018). There are a few other similar examples in the thesis.

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*