University of Rzeszow
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Institute of Biotechnology

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Review of the doctoral dissertation by

Ms. Natalia Łazarewicz, M.Sc.

entitled "Characterization of the nuclear ubiquitin ligases in the yeast *Saccharomyces cerevisiae*"

The doctoral dissertation, submitted for review by a PhD candidate Ms. Natalia Łazarewicz, M.Sc., was carried out under the supervision of Prof. dr hab. Robert Wysocki and dr hab. Gwenaël Rabut at the Department of Genetics and Cell Physiology, Faculty of Biological Sciences, University of Wroclaw, Poland and the Institute of Genetics and Development of Rennes (IGDR), University of Rennes, France, respectively, as a result of cotutelle agreement. The research performed and presented in the dissertation was funded by the National Science Center (Poland) PRELUDIUM 20 call, grant number 2021/41/N/NZ2/00551 entitled "The interactome of Upf1 (Nam7) ubiquitin ligase - deciphering the substrates and novel biological functions" for Ms. Natalia Łazarewicz as a Principal Investigator.

Ubiquitination, ubiquitin attachment to a target protein by the means of E1, E2 and E3 enzymatic activities, is one of the most abundant and important post-translational modification (PTM) modulating a plethora of physiological processes; for example, DNA replication, chromatin assembly, gene transcription, cell proliferation and cell death, cell trafficking, metabolism, immune responses, and development. Ubiquitination is a regulator of the stability, activity, localization, or binding partners of targeted substrates. Aberrant ubiquitination may result in mis-localization of proteins, accumulation of damaged or misfolded proteins, improper complex assembly, affected enzymatic and signaling pathway activities contributing to the development of human pathologies such as cancer, autoimmune diseases, developmental disorders, metabolic syndromes, and neurodegeneration. Despite the progress in the field of ubiquitination and related research, still little is known about the substrates and partners of numerous uncharacterized yet ubiquitin ligases (E3).

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The aim of the PhD thesis was to characterize the interactome and putative substrate proteins of poorly characterized ubiquitin ligases Nam7 and Irc20 using the budding yeast *Saccharomyces cerevisiae* as a model system. To address the aim of the study and draw final conclusions, the PhD candidate had developed and optimized a high-throughput up-to-date methodology to track ubiquitin ligase-mediated protein-protein interactions under physiological conditions, namely NanoLuc-based protein fragment complementation assay (PCA). In my opinion, the subject of the PhD thesis is very interesting, important, up-to-date and fully justified.

The dissertation of Ms. Natalia Łazarewicz, M.Sc., written in English, is a monograph and has 182 numbered pages. The layout of the dissertation is typical for experimental works. The dissertation is divided into the following chapters: abstract of the work prepared in Polish, French and English, list of abbreviations, introduction, purpose of the work, materials and methods, results, discussion, conclusions and perspectives, list of figures, list of tables, funding information, references, reference list for Table 2 and Table 4. The bibliography includes appropriately selected references in accordance with the topic of the work. The dissertation is also enriched with 50 figures and 15 tables.

The PhD thesis is not free of small language errors such as typos, incorrect punctation, editorial mistakes or improper use of words; however, the intention of the reviewer is not to provide a mistake list, as they are not very important for the understanding of the text and the interpretation of the results. Nevertheless, an incorrect use of Latin binomial nomenclature of selected organisms should be highlighted: *Escherichia coli* (for example, page 77), *Saccharomyces cerevisiae* (for example, page 148) or *Drosophila melanogaster* (for example, page 148) should be written in italic. This should be also applied to gene names, for example *UBI4* gene (page 23), *WWP1* gene (page 58), *HERC4* gene (page 58). "*In vitro*" is also not always consequently used in italic in the text (for example, page 130).

Evaluation of the scientific importance of the dissertation

In my opinion, **the title** *"Characterization of the nuclear ubiquitin ligases in the yeast Saccharomyces cerevisiae"* is too general. The title should be more specific. As the PhD

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candidate was mainly focused on Nam7 and Irc20 proteins, perhaps this information should be included in the title.

The introduction section is very comprehensive and well-written (46 pages in total). The PhD candidate provided information on the history of ubiquitination research with the emphasis of the mechanism, characterization of ubiquitin, types of ubiquitination and their biological roles, characterization of ubiquitin activating enzymes (E1), conjugating enzymes (E2), ubiquitin ligases (E3), ubiquitin chain elongation factor (E4), deubiquitinating enzymes (DUBs), the role of aberrant ubiquitination in human pathologies and characterization of methods for ubiquitination analysis such as protein-fragment complementation assays and NanoLuc-based luciferase assays. I have no major issues/concerns about this chapter. In my opinion, the Introduction contains all necessary information for the understanding of the content discussed later in the work. Graphical part is generally attractive to the readers; however, some tables (for example, Table 2, page 37) are hard to read (too small font size). Perhaps it would be also more convenient to read advantages and disadvantages of described techniques in a table with cited references. There is an incorrect numbering of some subsections, for example, "1.6.2 RING, 1.6.2.1 Irc20, 1.6.6 Nam7 (Upf1), 1.6.2.1 U-box" that may be quite confusing. "Figure 19B shows the dendrogram produced from the alignment of the first RING-like motif of yeast Nam7 with RINGs and U-box domains of other yeast proteins (page 41)." Perhaps it should be "Figure 12B"?

The purpose of the work was clearly defined in accordance with the dissertation topic. In addition, the PhD candidate specified two biological objectives. I have no comments to this part as well.

I would like to conclude that the stated scientific objectives fulfill the requirements for PhD thesis and the realization of these objectives significantly broadens the current knowledge on ubiquitination and provides the original approaches to solve a scientific problem.

The Materials and Methods section contains all necessary information for repeating the experiments by other researches. The catalog numbers of used reagents and kits, and the concentrations, if applicable, were provided. The protocols were comprehensively described. Numerous methods were applied that confirm the laboratory skills and experience of the PhD

candidate and allow for the verification of research hypothesis. For example, classical yeast biology methods, genetic engineering and molecular biology methods, and high-throughput fluorescence and luminescence assays were used. One drawback of this section is the lack of "statistical analysis" subsection. Except of "3.2.2.5 High-throughput data analysis", no information on statistical analysis is provided. I have a question about SD medium formulation (page 72); the PhD candidate stated that 20% glucose was used. According to Sherman F. Getting started with yeast. Methods Enzymol. 2002;350:3-41. doi: 10.1016/s0076-6879(02)50954-x, one should use 2% glucose. So, what was the rationale for using 20% glucose?

The obtained results are adequately described in **the Results section**. A lot of laboratory work was performed. For interactome part, five E3 ligases were selected (poorly characterized Irc20 and Nam7, and well-established Cdc53, Cdc4, and Met30 for comparison), and NanoBiT (NanoLuc® Binary Technology) PCA was used. To do so, yeast strains expressing five E3 ligases endogenously tagged with SmBiT (small BiT) at their C-terminus were crossed with a genome-wide collection of about 6000 strains expressing yeast ORFs endogenously tagged with LgBiT (large BiT) at their C-terminus. Upon crossing and sporulation steps, haploid strains expressing unique combinations of SmBiT and LgBiT tagged proteins were selected. The PhD candidate paid a great attention to optimization steps of genome-wide NanoBiT assays in the yeast model system. Fluorescein diacetate (FDA) staining was used to calculate the levels of viable metabolically active cells and correlate this parameter with luminescence measurements. Well-established E3 ligases were also used for interactome studies to validate NanoBiT-based approach. The full NanoLuc tagging was also used to reveal changes in protein abundance levels upon ligase inactivation under physiological conditions. Putative substrates of Irc20 and Nam7 were also evaluated using NUbiCA (NanoBiT-based ubiquitin conjugation assay). The results section is well-written and easy to follow. The description of obtained results is supplemented with the assessment of potential limitations of the study that highlights the scientific expertise and practical experience of the PhD candidate. In my opinion, the most important aspect is the optimization of high-throughput screens for specific and reproducible analysis of protein-protein interactions (PPI). These systems can be applied for other than ubiquitination research-related analysis of PPI in the future.

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The most important findings are: the correct identification of ubiquitin substrates for Cdc53 ubiquitin ligase based on NanoBiT and NanoLuc screens that confirms system validation; successful characterization of Nam7 interactome and identification of potential substrate proteins that might undergo degradation *via* Nam7; identification of novel Nam7 interactions with several E2-conjugating enzymes *in vivo*.

I have detected some issues with data presentation. Some figures are hard to read (for example, fig. 28B). A decimal fraction is denoted as "." or as ","; this should be unified (for example, fig. 19C). What was the rationale for providing some results twice (for example, fig. 28A and fig. 32)? Some figure legends are minimal and not informative, for example: "Fig. 47: Abundance level of different Nam7 mutants." No information on data analysis is provided. Some labels are not provided (for example, y axis), some scales are truncated (for example, y axis, fig. 39 for "TRA1"). Of course, my comments on data presentation do not limit the scientific importance of obtained data.

The obtained results were critically discussed **in the discussion section** in the light of available literature data. The PhD candidate interpreted the data with great caution. Technical problems are highlighted and data limitations are comprehensively analyzed. The PhD candidate discussed the problems with discrimination between the mRNA decay and a potential ligase functionality of Nam7, and low expression levels of Irc20 ligase. The problems with the identification of significant interactions (NanoBiT screen) and upregulated proteins (NanoLuc screen) are documented and some solutions are provided for further studies in the future, namely the verification of received Irc20 hits using the Irc20 overexpression system. Future directions are also highlighted in the section **Conclusions and Perspectives**. Perhaps for better readability, the final conclusions should be numbered and provided point-by-point. Furthermore, for better understanding of obtained data, a summarizing scheme/figure should be considered (a graphical summary in the discussion section).

After reading the chapters of results and discussion, I have several questions regarding the clarification of some aspects of the dissertation:

1. As optimization step was based on FDA staining for the assessment of cell viability, OD measurements and colony size analysis, did you also consider cell size analysis? Cell

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size may also interfere with fluorimetric and luminometric measurements in some experimental settings.

2. The PhD candidate stated (page 104): *"Unfortunately we were not able to identify either the Skp1 on Hrt1 protein among the interactome screen. This can be explained by the fact that the Skp1 protein contains the F-box domain at the C-terminus region and is not fully functional upon the C-terminal tagging. Also upon the selection of the haploid strains expressing Cdc53 fused with the SmBiT together with Skp1 tagged C-terminally with LgBiT the strains occurred to be lethal."* What are the consequences of the C-terminal tagging in terms of cell physiology? Did you analyze this issue? Did you observe cytotoxic effects in other constructs as well?
3. As the interactome analysis was performed under physiological conditions, did you consider to study the roles of Nam7 and Irc20 as potential ubiquitin ligases under genotoxic and proteotoxic stress conditions? What can be expected?
4. In the Introduction section, affected E3 ligase activity as an etiological factor of selected human diseases was described. It will be nice to discuss some practical aspects of obtained results, for example what are/can be biomedical implications of presented data? Taking into account that the budding yeast is a powerful model organism in terms of basic and applied research, what is known about the applications of yeast system to model human diseases associated with aberrant E3 ligase activity?

Conclusions

In summary, I would like to conclude that **the doctoral dissertation prepared by Ms. Natalia Łazarewicz, M.Sc., meets the requirements established in the Article No. 187 of the Act of 20 July 2018 on *"The Law on Higher Education and Science"* (i.e., The Journal of Laws 2023, item 742 with further amendments).** The PhD candidate has demonstrated that she is able to solve independently the identified scientific problems through well-planned experiments, data interpretation, critical discussion and adequate conclusions. The doctoral dissertation provides novel information to the discipline of biological sciences. Therefore, I recommend the Council of the Discipline of Biological Sciences of the University of Wrocław

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to continue the doctoral program procedures, i.e., PhD thesis defense by the PhD candidate Ms. Natalia Łazarewicz, M.Sc. Due to the high scientific importance of presented results on the development and optimalization of new high-throughput method for specific and reproducible analysis of protein-protein interaction based on NanoBiT screen in living cells, as well as obtaining a new information expanding the current state of knowledge in the field of ubiquitination research, I also recommend the Council of the Discipline of Biological Sciences of the University of Wrocław **to award the doctoral dissertation of Ms. Natalia Łazarewicz, M.Sc., as an outstanding dissertation (PhD thesis with honors).**

Sincerely yours

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