Skoltech
Skolkovo Institute of Science and Technology

Thesis Changes Log

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

Supervisors: Prof Timofei Zatsepin, Prof Konstantin Piatkov

Chair of PhD defense Jury: Prof. Yuri Kotelevtsev **Email**: *y.kotelevtsev@skoltech.ru*

Date of Thesis Defense: 03 September 2020

The thesis document includes the following changes in answer to the external review process.

I would like to thank all jury members for their thorough reading and correcting of my thesis. All comments and corrections have helped to make substantial improvements to the thesis, and I am grateful for their reviews.
As a result of their kind feedback, I have made several changes reported in detail below.

Professor Petr Sergiev

1. page 14 - "Figure should replaced with (most likely) "Figure 4"

Modification to the thesis:
This has been corrected to Figure 4.

2. page 17 - (Ling) incomplete citation

Modification to the thesis:
The correct citation was inserted.

3. page 62 - "BRCA1 containing the Asparagine residue" - likely "BRCA1 containing the Aspartate residue", if Asp is meant

Modification to the thesis:
Asparagine has been corrected to Aspartate.

4. Figure Downregulation of UBR4 the control siRNA treatment is puzzling. While explanation is provided, this point might require further clarification.

Response:
As with the mRNA levels, the observed downregulation of UBR4 proteins after chronic administration of LNP-ctrl was not observed in all experiments (see figure panel b below). Because we wanted to present all the data from the same experiment (mRNA and protein levels, TUNEL, weight, toxicity markers in the liver, etc), we kept the WB with UBR4 showing a decrease with the LNP-ctrl. Every batch of LNPs is slightly different, and this

particular batch of LNP-ctrl was bigger than usual by around 10 nm. This means more lipids, and more lipid accumulation in hepatocytes, which could trigger autophagy and trapping of UBR4 in autophagosomes, leading to its degradation.

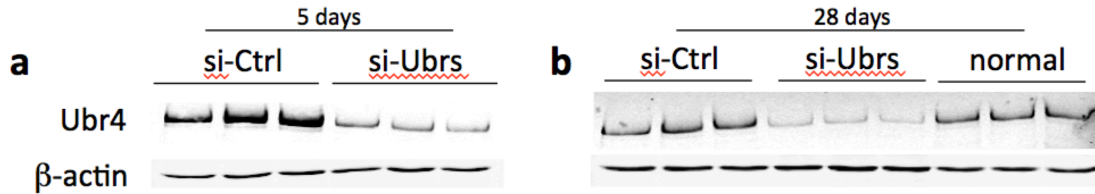


Figure**. UBR4 protein levels in the liver after siRNA treatment**. Mice were injected once (a) or 2x week for 4 weeks (b) with LNP-siRNA against Ubrs. LNP-siRNA against Luciferase were used as a control. Mice were killed on day 5 (a) or day 28 (b), and protein level was measured by Western Blot.

5. Figure 24b Panel labels are missed, likely to PBS, LNP Ctrl, LNP UBRs.

Modification to the thesis:

The labels have been added to Figure 24b.

6. page 76 The study of pro-inflammatory protein fragments accumulation would benefit from the clarification of several points. The approach taken is hypothesis driven, i.e. the predicted fragments are searched for and studied. lt might worthwhile to use approach to compare proteomes of control and UBR knockdown samples.

Response:

The author agrees that the study of the role of the Arg/N-degron pathway is only a beginning, and that many questions remain. However, both the concrete and circumstantial evidence provided by this study place the Arg/N-degron pathway as a player in the regulation of the inflammatory response. Further studies are needed to confirm more inflammatory fragments that are targeted for degradation via the N-degron pathway, and prove their degradation in a more physiological setting.

As for performing proteomic studies, this approach has been attempted many times in laboratories experienced with working with the N-degron pathway with mitigated success. Firstly, quantification techniques such as stable isotope labeling do not allow to distinguish between a full-length protein or its cleaved fragment. Therefore, accumulation of the stabilized fragment in a cell with a downregulated Arg/N-degron pathway would not be observed. Then, the interpretation of the results is challenging and deciphering between N-degron related events or consequences of apoptosis/cell stress onset is difficult. Turning off the N-degron pathway completely eventually induces apoptosis or increases ROS in cells or induces the misfolded protein response or initiates signaling pathways, all of which have important consequences on the proteome and will mask the accumulation of N-degron pathway targets and prevent their identification. Determination of the time-point to perform proteome analysis is also tricky: N-degron pathway substrates are quickly degraded, some even in minutes, while the siRNA-mediated approach takes days to downregulate the protein levels enough to have an impact on degradation rates. Finally, because many of the N-degrons require a cleavage event to be exposed, and because these cleavage events by activated caspases or endopeptidases also initiate a cascade of intracellular signaling that will change the proteome, following the stabilization of N-degron pathway substrates generally requires the use of artificial constructs where these substrates are tagged. Therefore, for these reasons, we chose not to use a proteomic

approach to study the consequences of Arg/N-degron pathway knockdown in the context of normal cells, cancer cells or in an inflammation context.

7. Figure 31 and its discussion monitor pro-inflammatory protein fragments accumulation upon UBRs inhibition an artificial approach was used, namely, construct coding for fusion protein, whose deubiquitination results in the formation of those fragments. Are these effects physiologically relevant? What share of endogenous proteins are indeed cleaved and whether an increase in fragments would have meaningful consequences? Would an increase in the amount of naturally formed fragments would sufficient to trigger any response?

Response:
As mentioned in the thesis, we examined protein fragments that are known targets of proinflammatory caspases or endopeptidases. This implies that the fragments are generated under inflammatory conditions. A detailed description of the fragments presented in the two first sections of table 7 of was added to the thesis. This new section lists the known proinflammatory roles of each of the fragments with confirmed or proposed N-degrons and should illustrate the physiological relevance of the cleavage event, the fragments themselves and how degradation of the fragments could participate in the regulation of inflammation.

Modification to the thesis:
A detailed description of the fragments presented in the two first sections of table 7 of was added to the thesis.

8. Figure 33 and its discussion. What is the identity of moderately shorter IL1 fragment? Where (in what compartment) the cleavage and degradation happen? Whether IL1 is located in ER/Golgi immediately after synthesis and whether ARG/N-degron pathway function in these compartments? Is the cleaved fragment functional? No decrease in full-length protein is obvious. Whether ectopic expression of cleaved form of IL1 have any phenotype?

Response:
IL-1β is first translated as pro-IL-1β, a 31kDa protein, and then processed into a 17kDa fragment, which is the mature active form. Caspase-1 also cleaves pro-IL-1β into a 26kDa form, whose function is unknown (Afonina et al, 2015). Cleavage occurs both in the cytoplasm and in the transport vesicles, and the location of the cleavage event depends on the stimulus. For instance, LPS generally causes cytosolic IL-1β processing. Secretion of IL-1β follows an unconventional route, most likely through autophagosome, microvesicle or exosome formation (Lopez-Castejon and Brough, 2011). Rab39a is required for the secretion of processed IL-1β and could be involved in the transport of the vesicles to the extracellular space. Rab39a is targeted for degradation by the N-degron pathway (IL-1β does not have an N-degron). We used the secretion of IL-1β as a read-out of the stabilization of Rab39a in the absence of a functioning Arg/N-degron pathway.
I suspect that the absence of decrease of the full-length protein is a question of timing, as IL-1β is translated after the LPS is administered and is continuously being produced. Most likely, a further time-point would show a decrease in the quantity of full-length protein.
I will be happy to further discuss this point during the defense.

9. page 107 on. lt is obvious to say that ARG/N-degron pathway function post-translationally and hence also post transcriptionally. Wouldn't it make sense to analyze difference between the intact and UBRS downregulated liver samples on PROTEOME level?

Response:

See answer to question 6.

Dr. Friedrich Felix Hoyer

1. Flow cytometry in Figure 23C and following figures. The candidate should consider to choose a more descriptive way to describe the macrophage population in the middle panel (such as CD11B-positive, Ly6C-positive myeloid cells instead of macrophages). Kupffer cells, which are the resident macrophages in the liver, do not by default express CD11B. Infiltrating, monocyte-derived macrophages may, however, transiently express CD11B. The population at hand is likely a mix of infiltrating monocytes (Ly6C-high) that differentiate into macrophages. It is unclear whether the candidate included macrophage markers such as CD64 or F4/80. I therefore recommend to omit the term "macrophages" and instead replace it by CD11Bhigh Ly6Chigh myeloid cells. Further, there are two gates in the middle panel, however, the author only refers to macrophages. Please remove the lower "intermediate" gate.

Response:
The markers CD64 and F4/80 were not included in the mix when interrogating cells in the spleen, however F4/80 was used to exclude Kupffer cells in the liver. The gating strategy was designed to exclude dendritic cells, eosinophils, neutrophils (and Kupffer cells in the liver) before gating on $CD11b^{high}/Ly6c^{high}/Ly6G^{-}$ cells. Therefore, although the author understands that there are various population of macrophages and that more markers would be needed to further characterize these cells, the choice was made to keep the "macrophage" label in the figures, for sake of simplicity, but to specify in the text that these are $CD11b^{high}/Ly6C^{high}$ expressing cells, most likely infiltrating monocytes differentiating into macrophages.
Modifications in the manuscript:
The intermediate gates were removed on the flow figures. The text was adjusted to better specify the type of cells.

2. The chosen lipid nanoparticles are taken up by hepatocytes, as the candidate clearly describes. Since there is significant accumulation in adipose tissue, the author speculates that peripheral macrophages take up the nanoparticle as well. The author should add a sentence on macrophage phagocytosis and lipid nanoparticles with subsequent implications for macrophage targeting. The chapter on RNAi and delivery methods is already fairly comprehensive. Yet, few information on lipid nanoparticles and macrophages would complete the picture.

Modifications in the manuscript:
The following paragraph was added:
While most cationic and ionizable lipid-based nanoparticle formulations deliver siRNA to the liver at more than 90% (Akinc et al. 2009; Love et al. 2010; Dong et al. 2014), some delivery to the kidney, spleen and lungs has been demonstrated. Additionally, professional phagocytic cells such as monocytes, macrophages and neutrophils can internalize nanoparticles while they are in circulation or in the spleen. In the case of the C12-200 ionizable lipid, delivery to cells of the immune system was demonstrated, and optimization of chemical composition of the LNPs increases this, although much higher doses are required compared to hepatocytes and less target gene downregulation is observed, indicating possible issues with accumulation in tissues with no uptake or endosomal escape (Novobrantseva et al. 2012; Leuschner et al. 2011). Indeed, if nanoparticles are phagocytosed, the internalized vesicles will fuse with lysosomes, leading to oligonucleotide destruction due to low pH (Gustafson et al. 2015). Therefore, fine-tuning of the lipid

nanoparticle components, including the selection of cationic or ionizable lipids, is needed for non-hepatic delivery, especially to myeloid cells (Whitehead et al. 2014).

3. The PhD candidate often uses the term "influences on" throughout the thesis. Please check every instance where you use this term and erase the word "on" where necessary.

Modifications in the manuscript:
The term "influences on" has been changed to "influences" where applicable.

Professor Emmanuelle Graciet

1. 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.

Modifications in the manuscript:
"n" values were added to all figures where applicable.

2. Chapter 1:

p2: very briefly indicate how sorafenib and regorafenib function. Why are they of relevance in this paragraph?
Modifications in the manuscript:
The text was modified as follows: The present standard-of-care for HCC includes multikinase inhibitors sorafenib and regorafenib, which inhibit cancer growth directly by causing tumor cell apoptosis, and indirectly by preventing angiogenesis in tumors. However, many patients respond poorly or develop resistance to sorafenib and only recently, multiple kinase-inhibitor lenvatinib and two different immune check-point inhibitors were approved as first-line treatment for unresectable HCC or for patients who progress after sorafenib.

3. 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).

Response:
Knockout of the ATE1 amounts to a partial ablation of the Arg/N-degron pathway, since not all substrates are arginylated. However, removal of all the E3 ligases of the pathway prevents recognition of all the substrates. To clarify, the sentence was modified in the text.
Modifications in the manuscript:
The sentence has been modified as follows: Second, since the lipid nanoparticles deliver siRNA only in the liver, we can study the role of the target proteins in this organ specifically, and we can also examine the consequences of downregulation of the Arg/N-degron pathway in adult tissue, which has never been done before.

4. 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.

Modifications in the manuscript:
The end of the paragraph was modified as follows:

The mechanism of transfer of the ubiquitin molecule onto the target protein is dependent on the type of E3, and this also affects the role of the E2 enzyme. For instance, if working with a HECT E3, the E2 enzyme will transfer the ubiquitin to the active site Cys of the E3, which will then transfer it to the target substrate. However for RING E3s, which function as co-factors for E2s, the E2 enzyme is responsible for determining which of the seven lysines of ubiquitin will serve for polyubiquitylation and catalytically transfers the ubiquitin molecule on the target protein (Stewart et al. 2016). Nonetheless, in all cases, the E3 enzymes are responsible for the specificity of the system by recognizing their own specific and unique substrates, and determining the identity of the lysine on the target protein (David et al. 2011; Mattiroli and Sixma 2014).

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

Modifications in the manuscript:

The reference has been added

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

Modifications in the manuscript:

The following paragraph was added to the preceding section:

An important part of protein regulation through the ubiquitin system is the capability of removing the ubiquitin molecule if the signal is no longer needed. Enzymes responsible for this process are called deubiquitylating enzymes (DUBs) and function by cleaving the amide bond between the ubiquitin and its substrate or the isopeptide bond between ubiquitin molecules. Through their biochemical activities, DUBs participate in ubiquitin precursor processing, editing or rescue of ubiquitin conjugates, protein trafficking, and are essential for developmental processes, for proper functioning of the cell cycle and various signaling events in the cell (Amerik and Hochstrasser 2004).

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

Modifications in the manuscript:

"N-terminal" has been added to the text.

8. 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.

Modifications in the manuscript:

A description of the proteins presented in Figure 2 (more specifically Gcn2, Fmt1, Cse4, Pgd1, Rps22a, Fbp1, Idl1, Mdh2, Pck1, Gid4, Doa10 and Not4) was added in the text following the figure.

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

Modifications in the manuscript:

Cys has been added to the list.

10. p13: Figure X??

Modifications in the manuscript:

Figure X has been replace by Figure 4.

11. p17: (Ling) - incomplete reference
Modifications in the manuscript:
The reference has been added.

12. 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.
Modifications in the manuscript:
The mention of "model plant" was added.

13. p35: BOX1 is extremely useful. It would be good to introduce it in the text with some details.
Modifications in the manuscript:
A reference to Box 1 was added in the section "Chemical modifications of siRNA", where efficacy and potency are first mentioned.

14. p62: Asp-BRCA1 starts with aspartic acid, not asparagine.
Modifications in the manuscript:
This has been corrected in the text.

15. Figure 17b: correct legend of y axis. This is quantitation of protein, not mRNA, so "expression" may not be the best term.
Modifications in the manuscript:
The word "expression" has been removed.

16. 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.
Response:
The difference between the sets is the identity of the siRNA. All other parameters of the experiments are the same when both sets were used.
Modifications in the manuscript:
A specific column was added to Table 5 to indicate which set the siRNA belong to. The following sentence was added as a footnote to table 5:
* In vitro experiments were performed using the set of siUbrs (1), unless specified otherwise

17. 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.
Modifications in the manuscript:
The results were changed as follows: Indeed, we observed a decrease of both cell proliferation and migration after transfection with si-UBRs compared to si-Ctrl although the effect on migration was not statistically significant (Figure 18).

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

Modifications in the manuscript:

The legend was changed to include the mention that one of two different siRNA per UBR (presented in Table 5) were injected per mouse. Other figures legends were reviewed and changed if both sets of siRNA were used, as Table 5 now indicates that set #1 was used throughout the study unless otherwise noted.

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

Modifications in the manuscript:

The following sentence was added to the text: Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were evaluated as indicators of liver toxicity.

20. Figure 27: the effect of LNP-siUbrs in HCC model is presented, with increased neutrophils and Ly6CHigh 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?

Response:

We did observe increased infiltration of immune cells in the livers and spleens of normal mice treated with LNP-Ubrs for 4 weeks, twice per week. These results are presented in figures 23 and 24 and discussed in the associated text. The magnitude of the increase is comparable for the $Ly6C^{high}$ macrophages, but is higher for neutrophils in the normal context.

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

Response:

The third section of Table 7 contains Caspase-1 substrates containing possible N-degrons, but with no experimentally confirmed proinflammatory roles.

Modifications in the manuscript:

A precision is now included as a title of the 3rd section of the table: Caspase-1 substrates containing possible N-degrons (no experimentally confirmed proinflammatory role)

22. 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.

Response:

Figures 30 and 31 present results obtained from rabbit reticulocyte extracts and are typically presented without error bars. However, they are representative of at least 2 independent experiments. A mention of this was added in the legend.

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

Response:

LPS is sufficient to activate caspase-1 in macrophages but ATP is needed for IL-1b secretion. For the experiment presented in Figure 34, only LPS was absolutely needed as we wanted to prove that UBRs are not cleaved by Caspase-1. However, our other experiments with

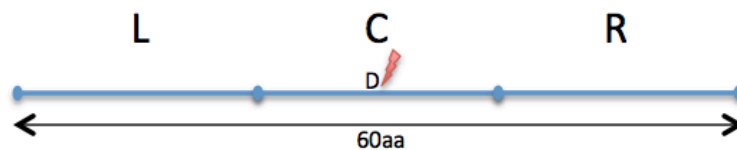
macrophages included ATP in order to observe IL-1b secretion. Therefore, the LPS+ATP condition was included, to replicate conditions used in previous experiments. The same result was expected between the LPS and LPS+ATP conditions, and indeed, the addition of ATP does not induce Caspase-1 to cleave UBRs.

24. 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.

Response:

The equation is original to the study and developed by our collaborators N Gubina and M Pyatkov The idea behind the formula is to determine the difference between the central part of the amino acid sequence of caspase substrates versus the lateral parts in terms of secondary structure or hydrophobicity. These calculations are empirical and based on the MUFOLD web-based program (for secondary structures) or hydrophobicity indices established by Radzicka & Wolfenden.

The question addressed by this equation is: is the central part of the 60-amino acid long sequence, (divided into 3 equal sections of 20 amino acids) different than the lateral sections?



? = Does C differ from L and R?

- In our conditions, C should differ from both L and R
- Therefore, we average them as (L+R)/2
- All three sections are equal in the number of amino acids
- The formula for calculation of the prevalence value is thus:

$$\frac{C}{(L+R)/2} \longrightarrow \frac{2C}{L+R}$$

If the value is above 1, then the central part is more likely to contain coils or to be hydrophobic than the lateral parts, and the opposite is true if the value is below 1.

Modifications in the manuscript:

The paragraph preceding the formula was modified as follows, to bring more precisions: We further developed the idea that caspase cleavage sites would be situated in regions that allow for the best accessibility. This implies that the sites could be situated between two structured regions, and between less hydrophilic regions, ensuring that the cleavage site would be exposed at the surface of the protein. For each human caspase substrate, we divided the 60-amino acid long sequences into three segments of 20 amino acids, with the central part containing the cleavage site, and analyzed the difference between the lateral environment of the cleavage site and the central part. For both the secondary structure and the composition of the amino acid sequences of caspase substrates, we attributed a prevalence value to the central 20 amino acid sequences surrounding the P1 aspartate over lateral 20 amino acid sequences using the following elaborated formula:

25. Table 10: unclear if potential substrates in this table are putative or experimentally

validated. Please clarify.

Modifications in the manuscript:

These are experimentally confirmed caspase substrates, but their role in apoptosis is putative. The mention has been added to the title of the table.

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

Modifications in the manuscript:

This sentence was removed as the justification for choosing UBR5 is clearly stated in the previous sentences. Eventually, the study of differentially regulated genes after knockdown of the other UBRs of the Arg/N-degron pathway should be performed to understand all genes affected by this pathway, but this will be in future work, and is stated as such in the conclusion of the thesis.

27. 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.

Response:

The author thanks the reviewer for this comment, and has reanalyzed the data first to produce Venn diagrams, but also to make sure that we did not overlook any genes. The numbers increased but only slightly. There are a few reasons why we could have obtained such a small number of DEGs. First, once we applied the significance threshold, we eliminated almost 98% of the genes in the list. We also applied strict criteria to the Fold change indices, eliminating another 90% of the remaining genes. Relaxing any of these two criteria would give a longer list of genes but would also reduce the significance and perhaps the biological relevance. Second, we chose a late time point, to be certain that the protein levels were as low as possible. However, it is possible that this time point was too late and that we missed some effects of UBR5 on gene transcription.

Modifications in the manuscript:

Several modifications were made to the manuscript following this comment:

1- Venn diagrams were added
2- Table 11 was reviewed
3- The pertaining results section was partially rewritten:

One of the interesting downregulated genes is SerpinA12, which is an apoptosis inhibitor, suppresses inflammation and modulates insulin action (Skonieczna et al. 2019; Zieger et al. 2018). Downregulation of SerpinA12 could be a complementary mechanism by which UBR5 increases apoptosis in hepatocytes. Another observation is that a large portion of the downregulated genes are involved in bile acid elimination and drug metabolism in the liver. This could imply either a direct regulation of these genes by UBR5 or a consequence of liver damage induced by the absence of UBR5 in the liver. The genes that were upregulated have roles in various cellular functions such as transport, cell proliferation, migration and death, transcription, histone formation, actin components and formation of secondary messengers.

4- The pertaining discussion section was partially rewritten:

The second set of targets examined is the list of genes that are differentially expressed after UBR5 knockdown in the liver of mice treated with LNP-siUBR5. Transcriptome analysis suggests that UBR5 may be involved in the regulation of sulfotransferase genes (from the Sult2a family) and a gene from the UDP glycosyltransferase 2 family. These genes are involved in bile acid elimination (Turgeon et al. 2001) and drug metabolism in the liver (Alnouti 2009). This could imply that UBR5 is involved in regulation of processes such as

detoxification from endogenous and xenobiotic substances, although further experiments are needed to confirm direct links between UBR5, Sult2a and Ugt2b. An additional interesting observation is the decrease in the expression of SerpinA12, which could be one of the mechanisms by which the Arg/N-degron pathway affects cell death and suppresses inflammation, as these two functions have been attributed to the VASPIN protein (Skonieczna et al. 2019; Zieger et al. 2018). Upregulated genes also tell an interesting story, although it remains to be explored if their increased expression is due to the absence of UBR5. The genes that were upregulated following UBR5 have a vast variety of functions: cell proliferation, transport of calcium or nucleosides, histone proteins, cell wall components, actin components and formation of diacylglycerol. Two of these genes, Slc28a1 and Ly6c1, are generally increased following proinflammatory cytokine signaling such as TNFα and IL-6, however no direct causative role has been discovered for these proteins in inflammation (Fernandez-Veledo et al. 2004; Lee, Wang, et al. 2013). Other genes are involved in cell proliferation, although their actions are contradictory: upregulation of Phlda2 has an antiproliferative effect (Wang et al. 2018), while Wnt11 promotes cell growth (Uysal-Onganer and Kypta 2012).

28. 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?

Response:

Shikonin promotes apoptosis through upregulation of RIPK1 and RIPK3. RIPK1 is a well known Arg/N-degron pathway substrate, and its stabilization through partial ablation of the Arg/N-degron pathway should cause sensitization to apoptosis-inducing drugs. In Agarwana and Banerjee 2016, the authors used the small molecule inhibitor RCF11 to partially block the action of the Arg/N-degron pathway, in combination with Shikonin to cause further accumulation of RIPK1 and RIPK3 and finally apoptosis in cancer cells. RCF11 has been demonstrated to bind to UBR1 and UBR5, but binding to UBR2 or UBR4 is yet to be published (Lee et al, 2008). Agarwana and Banerjee saw a distinct accumulation of RIPK1 in RCF11-treated cells, and not RIPK3, indicating that their inhibitor does inhibit the Arg/N-degron pathway. However, their experiment using a siRNA against UBR1 inhibited the action of RCF11 on cell viability, when in fact the effect should have been cumulative, or at least should have been the same as RCF11 alone. Nevertheless, the final results of the study do show a combinatorial effect of blocking the Arg/N-degron pathway with an apoptosis-inducing drug. This could suggest that UBR1 and UBR5 are the E3 ligases responsible for targeted degradation of RIPK1, or that in this particular model, inhibition of the action of these two E3 ligases is sufficient, or that RCF11 does in fact bind to UBR2 and UBR4 as well. In conclusion, the results of the study by Agarwana and Banerjee concur with our results in that targeting the Arg/N-degron pathway potentiates the action of chemotherapy, no matter the specific mechanism of action of the chemotherapy.

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

Response:

The author thanks the reviewer for this suggestion and agrees that such information would be interesting. However, to present something comprehensive in the context of this thesis, the author would need to talk about expression patters of all four UBR ubiquitin ligases in many different tissues of human and mice, at the gene and protein levels. This is a substantial amount of information and could burden the text. Therefore, the author prefers to refer the reader to the proper references.

30. Formatting issues to address:
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.
Modifications in the manuscript:
The notation for decimals has been changed to use the dot.

31. Some typos left.
Modifications in the manuscript:
These have been corrected

32. Some mentions of N-end rule pathway instead of N-degron pathway.
Modifications in the manuscript:
This has been corrected to "N-degron pathway".

33. Some abbreviations are not defined the first time they are used but later in the text.
Modifications in the manuscript:
Abbreviations have been revised in the text

34. "micro" indicated with a u instead of Greek letter for mu.
Modifications in the manuscript:
The Greek letter mu was used to replace 'u" where applicable.

35. 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.
Modifications in the manuscript:
The references have been revised.

Professor Konstantin Lukyanov

1. Abstract: Please explain "HCC" abbreviation here (in addition to the List of abbreviations).
Modifications in the manuscript:
HCC has been written in full length.

2. Page 13: "The study by Tasaki and colleagues revealed seven E3 ligases bearing the UBR box (Figure x)...".
Modifications in the manuscript:
Figure x has been replaced by Figure 4.

3. Fig.19: Please describe the scale bar in the legend.
Modifications in the manuscript:
The scale bar measurements have been added.

4. Page 59: "...we designed 10 19-mer siRNA sequences per gene and ranked them based on their possible off target recognition and known miRNA and immune stimulatory sequence motifs ... The 10 best scored siRNA against each UBR ubiquitin ligase (Table 2) were screened ...". From this description, it follows that you selected the best 10 out of 10 siRNAs; so probably there is some misprint in numbers.

Modifications in the manuscript:
The sentence has been rephrased to read: …we designed 19-mer siRNA sequences per gene and ranked them based on their possible off target recognition and known miRNA and immune stimulatory sequence motifs ...

5. Page 62: "This reporter system is comprised of a ubiquitin molecule fused to the N-terminus of a reference FLAG-tagged derivative of the mouse dihydrofolate reductase (fDHFR-UbR48) coupled to FLAG-tagged BRCA1, a known target of the Arg/N-degron pathway (see Figure 29 for illustration)". This description seems to be different from what is depicted in Fig. 29 (also, it is inconvenient to go far away through the dissertation to look at the Figure). Please add a scheme of the reporter as a panel in Fig. 17.

Modifications in the manuscript:
A scheme of the reporter was added to Figure 17.

6. Page 62: "Cotranslational cleavage deubiquitylases produces ...". Preposition "by" is missed.

Modifications in the manuscript:
The preposition "by" was added.

7. Page 62-63: "we observed a decrease of both cell proliferation and migration after transfection with si-UBRs compared to si-Ctrl (Figure 18)". In contrast, Fig. 18b shows "non-significant" differences in migration rates (although values for si-Ubrs(1) and si-Ubrs(2) were clearly lower than in control samples). Please comment.

Response:
The migration assay is highly sensitive to variations in cell health, proliferation, plating, etc. Although we could see a difference in migration with the naked eye between cells transfected with siRNA against UBRs or with the control siRNA in every experiment, the variability between biological replicates was such that statistical significance was not reached. The difference was detectable, but non-significant.

8. Page 63: "... confirming the on-target effect of siRNA mediated downregulation on cell proliferation, migration and apoptosis". As the experiments showed upregulation of apoptosis (Fig. 19), it should be "... confirming the on-target effect of siRNA mediated downregulation on cell proliferation and migration and upregulation of apoptosis.".

Modifications in the manuscript:
The sentence was modified as follows: …confirming the on-target effect of siRNA mediated downregulation of UBR ubiquitin ligases on cell proliferation, migration and apoptosis

9. Page 67: "Maximal mRNA and protein downregulation in the liver occurred 3 days after injection, followed by a slow recovery (Figure 21c)". In fact, data are available starting only from the 3rd day for mRNAs and the 5th day for proteins. Potentially, maximal downregulation can occur earlier, the authors just have no data on this. In addition, there are no detectable "slow recovery" of UBR1 protein in the graph. Please rephrase this sentence to describe these results more accurately.

Modifications in the manuscript:
The author agrees on this comment, and has rephrased as follows: The maximal observed mRNA and protein downregulation in the liver occurred at the first time point examined (72h), (Figure 21c), and silencing to more than 60% lasted at least 10 days, which allows a

convenient once-per-week regimen for multiple dosing.

10. Page 76: A line break within the sentence "... Leu680-Matrin-3 (Table 7)."
Modifications in the manuscript:
This formatting issue was solved in the latest version of the thesis.

11. Table 11: "Espnl – Actin component". I would suggest changing to "Actin cytoskeleton component".
Modifications in the manuscript:
"Cytoskeleton" has been added to Espnl.

12. Ideally, "Conclusion" section should additionally contain a concise list of the main findings – proven experimental facts demonstrated in this work for the first time.
Modifications in the manuscript:
The following list was added to the conclusion section:
The conclusions to this study are three-fold and can be summarized as follows:

- The Arg/N-degron pathway was validated as a promising target for cancer therapy and potentiates the action of chemotherapy.
- The Arg/N-degron pathway plays an important role in the regulation of inflammation through targeted degradation of proinflammatory substrates
- This study led to the identification of potential partners for co-therapy, to further sensitize cells to apoptosis

Professor Yuri Kotelevtsev

1. Obviously as in every serious study there are open questions still to answer. The proinflammatory effects of Ubr knockdown require further explanation, particularly the generalized effect described in the spleen and pancreas. Mechanistic investigation targeting candidate proinflammatory pathways, cytokines, transcription factors affected by N-Arg-degron warrants further investigation. In this respect some mechanisms evaluated in the discussion require experimental support.

Response:
The author thanks the reviewer for his comments, and is looking forward to discussions during the thesis defense.