Posudek oponenta disertační práce/Opponents review

Studijní program:	Molekulární a buněčná biologie, genetika a virologie
Study programme:	*Molecular and Cellular Biology, Genetics and Virology*
Student:	**Mgr. Tomáš Liďák**
Školitel/Supervisor:	Mgr. Lukáš Čermák, Ph.D.
Disertační práce:	Identifikace a funkční charakterizace nových substrátů cullin-RING ubiquitin ligáz
Doctoral thesis:	Novel substrates of cullin-RING ubiquitin ligases: identification and functional characterisation
Oponent/Opponent:	Mgr. Jan Mašek, PhD Dept. Of Cellular Biology, Faculty of Science, Viničná 7, Charles University, Praha

Text posudku/Review:

The presented thesis focuses on the biology of cullin-RING ubiquitin ligase 4 (CRL4) subfamily members $CRL4^{DCAF4}$ and $CRL4^{DCAF12}$. Ubiquitin ligases are protein complexes that constantly regulate cellular protein turnover in all eukaryotes. Participating in every aspect of homeostasis, the deregulation of ligase activity often leads to cancer and other diseases. A key step of the process lies in the correct recognition of their target and attachment of a Ubiquitin molecule(s), directing the protein to degradation in the proteasome. The substrate recognition relies on the variable substrate targeting molecule of the E3 ligase that associates with the protein scaffold that mediates the activity of the catalytical E2 subunit. Hundreds of E3 ligases were discovered, however, very little is known about individual substrate specificities. In the case of $CRL4^{DCAF4}$ and $CRL4^{DCAF12}$ are the DCAF4 and DCAF12 are responsible for substrate recognition, but their targets were, until now, unknown.

The theoretical introduction of the thesis provides an extensive overview of the ubiquitin ligase systems molecular mechanisms, and their roles in homeostasis and disease, which is nicely written, and well referenced. This part is followed by an equally detailed review of the diverse biological functions of the $CRL4^{DCAF4}$ and $CRL4^{DCAF12}$ newly identified targets, unfortunately without clearly stated reason for their introduction which makes reading difficult as the reader needs to jump across complex topics of telomerases, apoptosis, spermatogenesis, riboproteins, and immunology in quick succession, and little idea of the authors' underlying intention.

The objectives of the thesis are clearly stated, but very ambitious, as they aim at the identification of both DCAF4 and DCAF12 targets, their validation, and characterisation of not only the mechanism of interaction with the E3 ligase but also the upstream inducers of the degradation and functional characterisation of the $CRL4^{DCAF4}$ and $CRL4^{DCAF12}$ role in vivo.

These questions are solved by appropriate and impressively diverse methodology encompassing mass spectrometry experiments, immunoprecipitation, affinity purification, WB, ELISA, fluorescent microscopy, flow cytometry, and qPCR performed in multiple cell line systems modified by CRISPR/Cas9 technology and retroviral transductions and whole-body KO mouse model (in case of DCAF12).

Collectively, these approaches led to the identification and validation of several targets of $CRL4^{DCAF4}$. Specifically, multiple components of the H/ACA Riboprotein - DKC1, GAR1, NHP2 – that are bound by $CRL4^{DCAF4}$ possibly with help of NOD10. This interaction is more efficient without the RNA. -

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Even more potential substrates were found for $CRL4^{DCAF12}$, out of which XIAP, SMAC and MOV10 were studied in more detail. Further experiments lead to the identification of the BIR2 and BIR3 domains of XIAP being necessary for the interaction of XIAP and SMAC with DCAF12. An interesting observation was also the cytoplasm restricted MOV10 degradation, which is dependent on its c-terminal -EL motif. The in vivo part of the study using DCAF12 KO mouse revealed reduction of the sperm counts, splenic counts of T cells and altered ratio of NKT cells and CD4+ T cells, in the absence of DCAF12, possibly mediated by the elevated levels of MOV10.

All presented data are valuable for the UPS research community and lay a substantial basis for multiple future studies. Unfortunately, the impact of the data and their interpretation is hindered by missing indicators of the data reproducibility, namely:

A) There is no indication of the number of experimental replicates, densitometry quantifications, nor a consistent display of loading controls (Fig. 7, 18) for the WB based experiments. Fig. 10, and half of Fig. 20a presents bar graphs without error bars. Fig. 22 and related text does not show P-value and claims significance (also in the published article). Fig 24 is based on two values only and does not allow for drawing any conclusions.
B) The newly developed DCAF12 KO mouse strain is not validated in any way (WB, immunofluorescence, Sanger sequencing, qPCR), strikingly it is the same also in the published article in the Special issue of MBPI International Journal of Molecular Sciences.
C) Immunoprecipitations in Fig. 8, 16 are lacking the WCL controls.

With exception of the three major methodological flaws, the thesis represents a complex testimony of the candidate‘s prowess in experimental design, mature scientific writing and numerous techniques of cell and molecular biology. Given a substantial erratum of the points A), B), and C) is provided, I am more than happy to recommend Mgr. Liďák’ s candidature for PhD degree.

Questions:

1) In Fig. 6, 12, 16, 17 - Some precipitated proteins are not present or barely detectable in WCL (often DDB1, MOV10 in some). What is your explanation?
2) In several of the experiments with DOX inducible DCAF12 construct expression Fig 14a, 15e, 21d are levels of expression very variable in between the different cell lines/conditions. How do you interpret this variability?
3) In Fig. 22 author works nicely with the publicly available MCA resource which allows also for the assessment of mRNA expression in various stages of mouse development. Have you considered looking into the stage-dependent expression of the DCAF12 and MOV10 biology?
4) You nicely show the altered T cell populations in the adult DCAF12 KO mouse. Also experiments with T cells purified from the DCAF12 KO mouse model suggest the mice might be immunocompromised, could you think of a different approach of how to test this in vivo?
5) How big do you think is the substrate redundancy of E3 ligases or the cullin/RING ligases - any biological reasoning behind it?

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