INSTITUTE OF EXPERIMENTAL ENDOCRINOROLOGY

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Review

of the **Matej Horváth**'s PhD thesis "A role of Sirt1 in the Notch signalling pathway"

Notch (*N*) signaling is one of the most intensely studied pathway that was discovered in a model organism of *Drosophila*, and found to be evolutionary conserved in numerous metazoans, including humans. It plays crucial role in multitude of cellular processes during embryonic and postembryonic development such as decision making between alternative cell fates by the well-known process of „lateral inhibition" or between two sister cells, by a process called „lineage decision". The another important role of Notch pathway is in formation of cell boundaries within various tissues. Human/mammalian orthologues of *Notch* gene are known to be implicated in several diseases, including cancer, and therefore studying this signaling pathway has always a good potential to add further information in many cellular processes during normal development and disease-prone aspects of human pathology.

The PhD dissertation of Matej Horváth is composed of major obligatory chapters, and is presented on 112 pages. The major portion of the text is devoted to Introduction which represents almost exhausting view of historical and mainly current and up-to-date literature on the N signaling, on N pathway members and their action in development. On one side this chapter is extraordinarily long in comparison to rest of the dissertation, however, as reader I would like to appreciate systemically and properly processed references with respect to the topics of the dissertation, and thus its further reading, notably Results, makes it easier and well understandable.

Author has divided its experimental interest into 5 major problems:

1. To investigate if Notch signalling pathway is sensitive to changes in basal metabolism in *Drosophila.*

2. To investigate if Notch signalling sensitivity to changes in basal metabolism is mediated by Sirt1.

3. To identify what part of the interactome do Notch and Sirt1 have in common.

4. To deduce possible Sirt1 substrates that can regulate Notch response.

5. To investigate the role of Sirt1 in Notch regulated developmental processes.

The battery of methods used in the course of the study is also impressive, although author devoted to the Materials and Methods description only 6 pages, and I am positive that in the form of dissertation it would devote more attention, specifically some details, as it may be

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apparent from some of my comments and questions below. Overally, work is composed of independent *in vitro* and *in vivo* experiments which nicely complements each other, and provide better and clearer picture of the N and Sirt1 action.

Mgr. Matej Horváth in his dissertation examined the role of Sirt1 in three models associated with Notch pathway: 1. Metabolically stressed S2 cells stably expressing full length wild type Notch receptor under inducible promoter (S2N cells). 2. Sensory organ precursor specifications and scutellar bristle development. 3. Wing D/V boundary formation and wing vein development. In all three models, author was able to demonstrate that Sirt1 positively influences the Notch pathway, what has not been the outcome expected on the basis of a literature. Sirt1 participates in the modulation of the expression of a subset of *E(Spl)* genes in metabolically stressed S2N cells and shows positive genetic interaction with main Notch signalling members such as Notch receptor, Delta ligand and repressor of Notch signalling, Hairless.

To further examine role of Sirt1 in Notch signalling Matej performed proteomic analysis of Sirt1 associated proteins in *Drosophila* embryos and in S2N cells. Surprisingly, Sirt1 was found in both with activator and repressor complexes involved in Notch target genes regulation, suggesting a direct link between Sirt1 and the Notch pathway. In addition, modulating Sirt1 activity in Hi5 cells, using chemical activators and inhibitors, showed that acetylation status of the Su(H) is controlled via Sirt1 activity. Author was able to show that association of Sirt1 with N^{ICD} and Su(H) is increased after Notch pathway activation, suggesting more exclusive role of Sirt1 in this signaling.

It should be highlighted that Matej Horváth, and his co-workers, of course, were first to demonstrate three new context-dependent findings about Sirt1, never shown before:
1. Activity od Sirt1 is inhibited by 2-deoxyglucose in *Drosophila* embryonic tissue culture.
2. Sirt1 works as a positive regulator of Notch signalling in *Drosophila*.
3. Sirt1 participates in regulation of acetylation status of Su(H).

The aim of the thesis was to examine role of Sirt1 in the Notch signalling pathway, using *Drosophila* as a model organism. Based on *in vivo* and *in vitro* studies, author concludes that Sirt1 plays a positive role in Notch signalling. In embryonic S2N cells, Sirt1 is responsible for the protection from metabolic stress-induced down-regulation of subset of *E(Spl)* genes. During development, Sirt1 was found to be responsible for proper Notch-dependent specification of SOPs and wing development. Sirt1 can regulate the Notch signalling on multiple levels via deacetylation of various substrates involved in the Notch signalling revealed by the proteomic survey.

Altogether obtained results are presented in logical sequence, the PhD dissertation is written simply and concisely, and to be easily understood. In spite of that there are quite numerous albeit little formal errors and typos in the text. Headings of some chapters were page-centered although majority of headings and chapter's titles were right-justified, what

should be considered correct. With respect to the genetic nomenclature, so well preserved and maintained in *Drosophila* community, there was little attention paid throughout the dissertation to correct usage of italics for original gene or allele names like E(Spl) *vs.* *E(Spl)*,

Su(H) *vs.* *Su(H)*, Sirt1 *vs.* *Sirt1* *etc.* Furthermore, reading author's paper and thesis rises some questions or points of interest which I feel to be addressed, and answered, as follows:

[1] Although the use of 2-deoxyglucose in metabolic-related studies is logical, to make any conclusion on real respiratory activity, especially in relation to *Sirt1* or *N* genetic background would require direct respiratory (physiological) assessment and/or measuring activity of key enzymes in mitochondrial respiratory chain. Author has even mentioned some respiratory measurements (pages 52 and 73), but did not present these data in the dissertation. Could you please comment on this ?

[2] From Figure 16 on page 56 it appears to me that *Sirt1* RNAi could work less efficiently than expected, and thus it might have effects on the experimental outcome and discussed results on mediation of metabolic sensitivity of *E(Spl)* gene mediated by Sirt1, which is supported also by the observation that *N*-dependent response of some genes treated with Ex527 under 2-deoxyglucose was lower than after *Sirt1* RNAi (page 58). Why prolonged, or repeated *Sirt1* RNAi knockdown was not performed to try to remove *Sirt1* RNA completely ? Was there any specific reason not to do so ?

[3] Would it be too speculative to ask whether results on 2-deoxyglucose you present can be considered that 2-DG has rate-limiting effects on Sirt1 function ? If you have different view, please comment on it.

[4] On pages 57 and 72 you mentioned that Sirt1 participates in the modulation of the expression of a subset of *E(Spl)* genes in metabolically stressed S2N cells and/or that Sirt1 is responsible for the protection from metabolic stress-induced down-regulation of subset of *E(Spl)* genes. These are very interesting results. Do you have clue why it is so, and whether is a chance to find what *E(Spl)* genes inside a particular subset have in common, or what differs between these two subsets of *E(Spl)* genes ?

[5] Your proteomic data on Sirt1-interacting partners are really very robust, and these would definitely deserve wider discussion, which is not validable under this format. However, I had simple question about commercial d-300 antibody used in this study. Both DSHB monoclonal antibodies are well known and defined, however, I was unable to identify d-300 antibody from Santa Cruz Biotech. Company has only cyclin B rabbit polyclonal antibody under designation d-300, or then H-300 polyclonal antibody but against human/mouse Sirt1 not *Drosophila* Sirt1. Could you explain this, please ?

[6] Regarding your interaction study between Sirt1 and Su(H), on page 68 it is mentioned that „Su(H) protein had an amino acid composition that makes it resistant to the digest with common proteases used for MS analysis". On one side I understand that there is a need to explain how some, even abundant, proteins could be missing from MS analysis (we do have

very similar experience with other *Drosophila* proteins too), however, was the meaning of this sentence based on repeated experimental evidence ? Simple *in silico* mapping of Su(H) protein provides numerous cleavage or digestion sites for trypsin, chymotrypsin, proline endopeptidase or Staphylococcal peptidase I (all used in MS studies), in addition to CnBr or NH_2OH sites.

Based on above mentioned facts, generally accepted international requirements for PhD theses, author's contribution of the research subject with obtained evidence, experimental methods used, the achievements of Mgr. Matej Horváth appears to meet acceptable scholarly standards for PhD dissertation. Therefore, I can conclude that he fulfilled all major stipulations, and I can gladly recommend the thesis for the defense in the front of a PhD committee, and if such a right is allowed to the reviewer, I would advocate for very good grading.

Bratislava 16. 8. 2017

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14/8/2017

Report on PhD thesis of Mgr. Matej Horvath.

The report describes the role of Sirtuin protein 1 in the regulation of Notch signalling using the *Drosophila* model system.

The introduction was well-written, comprehensive, detailed and accurate, covering all necessary areas of the literature spanning Notch structure function and activation, developmental roles, covalent modification. The basics of metabolism and its control required to understand and interpret the project were also well covered and with the review eventually leading to a focus on aspects directly related to the project in question i.e. the sirtuins and their cellular functions. The aims on page 43 are a little brief and could do with a some sentences for each point explaining further the rationale. If there is any preliminary data or prior work linking sirt1 to Notch other than an educated hypothesis then this should be made more clear at this stage.

Methods are well written and sufficiently comprehensive and procedures used for statistical analysis of phenotypic quantifications are included.

Results and discussion are nicely written and preliminary finding are used to produce a working model which is subsequently tested in later sections. Experiments were performed both with RNAi and with chemical inhibitors to produce similar conclusions, increasing confidence in the outcomes. A multidisciplinary approach combining methods of signalling assays, gene expression measurements, genetic interactions *in vivo* and protein-protein interactions makes for nice study.

I have just a small number of queries which might be brought up at the viva.
On p51 , high levels of Espl mbeta and M3 expression are seen is S2 N cells without EDTA treatment. This is curious and I am wondering if activation of expression of these genes actually Notch dependent in these conditions? Were target genes quantified in s2 cells not expressing Notch as a baseline control ?

How do we know that metabolism is actually affected in the s2 cells in this experiment? Could the unpublished data regarding this quoted on p52 be elaborated on?

On p53, how do we know primers are specific for target genes? What checks were made for what is actually being amplified?

On p65 with so many interacting partners of Sirt1 is it surprising that the Sirt 1 knock down phenotypes in the fly (p61 and 65) appear to be quite Notch specific?

A general question how could the study be improved given more time in order to arrive at a more specific molecular model of action?

Overall an excellent thesis with a very nice accompanying published paper. I recommend the report proceed to a *viva* defense and I would suggest a preliminary score of 1 subject to confirmation by *viva* performance.

Yours sincerely,

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Hohenheim, 1. August 2017

Evaluation of PhD thesis of Matej Horváth

Title of thesis: *A role of Sirt1 in the Notch signalling pathway*

to be presented for the committee for PhD studies in Molecular and Cell Biology, Faculty of Science, University of South Bohemia

The spatiotemporal development of multicellular organisms is governed by a handful of signalling cascades, which are well conserved in higher organisms including humans. The Notch signalling pathway belongs to this category of transduction cascades, being involved in a multitude of cell fate decisions by mediating direct cell-cell communication. Not surprisingly, dysregulation of Notch activity is a major cause of various diseases, including solid tumours and leukemias. Deciphering the levels and mechanisms of Notch signal regulation is key to understanding the context specificity of this pathway during development and disease. The serious consequences of aberrant Notch signalling have triggered large-scale efforts aimed at understanding the regulatory mechanisms underlying Notch activity. One hallmark of these studies is that regulatory mechanisms can occur at multiple steps of signal transduction. Such investigative studies, however, are complicated by genetic redundancy in vertebrates, *e.g.* mammals have four Notch receptors and five ligands, highlighting the importance of using model organisms like *Drosophila melanogaster* to elucidate novel mechanisms of regulation.

The principles of Notch signal transduction *per se* are rather simple as it operates via direct contact between signal sending and signal receiving cell: Binding of the ligand to the extracellular part of the Notch receptor triggers its cleavage releasing the intracellular domain of Notch (NICD) in the signal receiving cell. NICD shuttles to the nucleus and assembles a

ternary activator complex with the transcription factor CSL (CBF-1/Suppressor of Hairless/Lag-1). If Notch signalling is not activated CSL acts as molecular switch by assembling repressor complexes.

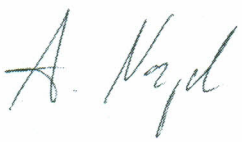
In his PhD work, Matej Horváth has carried out some pioneering research that connects the protein deacetylase Sirt1 with Notch signalling activity opening a new avenue of Notch signal regulation in *Drosophila*. In a first approach, he used S2 cell culture lines to show that several well-defined N target genes are sensitive to changes in basal metabolism of the cell and that this sensitivity is mediated by Sirt1 in a positive manner. Initially, these results were astonishing, as data from vertebrates mainly point to a negative interplay of Sirtuins and Notch signalling activity. However, Matej Horváth provides further compelling evidence for a positive connection by performing genetic interaction studies during bristle and wing development of the fly. In order to elucidate the molecular mechanism of the Sirt1-Notch connection in more detail he conducted mass spectrometry analysis based on embryonic nuclear extracts. Interestingly, he could find Sirt1 in both, activator and repressor complexes known to regulate Notch signalling activity, suggesting a direct, albeit complex link between Sirt1 and the Notch pathway. In the last part of his thesis he validated some of this uncovered interactors by performing co-immunoprecipitation and genetic interaction assays. Most interestingly, Matej identified amongst other proteins also the central Notch transducer Suppressor of Hairless [Su(H)] to be associated with Sirt1 and provide further molecular evidence that Su(H) is a direct target of the deacetylase Sirt1.

The PhD thesis is well written and presented. The introduction is very elaborated leaving the reader with the feeling to be up to date with the actual state of knowledge regarding Notch signalling regulation and metabolism in *Drosophila* as well as vertebrates. The results section of his work is well structured and the experiments have been carefully executed and presented. Matej presented several techniques in his thesis that demonstrate the large repertoire of methods he has learnt during his PhD training. I also want to emphasize his internship at the Erasmus medical Centre in Rotterdam, which was funded by an EMBO travelling grant allowing him to conduct the mass spectrometry analyses. The discussion clearly demonstrates his ability to critically scrutinise his results. Acknowledgement of his contribution to the field of Notch signalling regulation in *Drosophila* has already been made as he is the first author of a manuscript published in the high ranking 'Biochemical Journal' 2016.

For these reasons, and based on the high quality of the presented thesis, I recommend that the Faculty of Science at the University of South Bohemia accept this thesis, and grade it with:

excellent - very good

Sincerely Yours



(appl Prof. Dr. Anja Nagel)

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Hohenheim, 1. August 2017

Suggested questions for defend of thesis Matej Horváth

A role of Sirt1 in the Notch signalling pathway

to be asked by the committee for PhD studies in Molecular and Cell Biology, Faculty of Science, University of South Bohemia

1.) Immunoprecipitation gives hint, whether proteins can be found together in a protein complex or not. However, a direct interaction between two proteins cannot be guaranteed with this experimental setup. Which experiments could be done to show, that Sirt1 and Su(H) ***directly*** interact with each other? Which experimental setup would show that Sirt1 can be detected at the regulatory region of Notch target genes (together with Su(H))?

2. How is deacetylation of proteins connected with protein stability? What is known about the protein stability of Suppressor of Hairless in *Drosophila*?

3. How would you proceed to identify the lysine(s) deacetylated by Sirt1 in Suppressor of Hairless? What kind of phenotype would you expect from a *Su(H)* mutant which can no longer be deacetylated by Sirt1?

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Evaluation of PhD thesis of Matej Horváth

A role of Sirt1 in the Notch signalling pathway

Presented PhD thesis is devoted to the study of the role of Sirt1 deacetylase in Notch signalling pathway. In the introduction is described Notch signalling pathway and its role during *Drosophila* development, post-translational modifications of Notch signalling pathway components and their role in the response of this pathway to stimuli, regulation of Notch signalling by basal metabolism. I positively evaluate the sub-chapter clearly describing basal metabolism. Similarly, chapter concerning the Sirt1 function and regulation is written in a clear and comprehensible manner.

To fulfil the set goals, Matej has used number of biochemical, molecular and genetic methods, which he described in detail in the chapter Materials and Methods. In this chapter, I was missing description of creation of MARCM clones and Drosophila lines used in this experiment, although the results are only listed as supplementary.

The author has used S2 cell culture to demonstrate that some Notch target genes are sensitive to changes in basal metabolism and Sirt1 is required for proper expression of several E(Spl) genes. Using Sirt1 deacetylase activators and inhibitors he has shown that the metabolic sensitivity of E(Spl) genes is mediated by Sirt1 and that under conditions of metabolic stress activity of Sirt1 is inhibited. Series of genetic experiments confirmed the positive effect of Sirt1 on Notch signalling during Drosophila macrochaete and wing development. Using mass spectroscopy based on embryonic protein extracts he revealed number of Sirt1 interacting proteins among which were components of Notch signalling pathway as well as activators and repressors of this signalling pathway. Based on the other molecular method he has shown direct interaction between Su(H) and Sirt1 and that Su(H) is the potential substrate for Sirt1 deacetylase.

The results were sufficiently discussed and compared with the up to date literature.

Overall, the work is written in a comprehensible form without serious deficiencies, perhaps with the exception of some figures, such as Figure 20 and Figure 21, which could be larger for better orientation in the results.

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I have several questions for Matej:

In the literature review you are mentioning that almost all Notch receptors in *Drosophila* presented on cell membrane are in full length form, however when you mutate Furin cleavage site on Notch receptor you can see Notch loss of function phenotype. Therefore, I am wondering, what is the phenotype of Furin mutants?

Do you have any candidates besides Sirt1, which can also work as metabolic sensors for Notch signalling?

Sirt1 mutant has Notch loss of function phenotype manifesting in extra scutellar bristles and vein deltas in wings. Have you observed any other phenotypes besides these two?

You are mentioning that *Drosophila* contains four more, is it possible that one/some of them can complement Sirt1 loss of function? Have you tried to create Sirt1 mutant flies in combination with other sirtuin mutants? What was the phenotype?

Based on the quality of the presented thesis I recommend the committee to accept this thesis and grade it with: Excellent.

V Bratislave, 21.08.2017

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