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To: Prof Miroslav Obornik,
Head of the Committee for Ph.D. studies, University of South Bohemia Oct 5, 2016
Re: Review of the Ph.D. thesis submitted by Vaclav Broz.

Dear Professor Obornik,
I have reviewed the thesis submitted by Vaclav Broz entitled, " Role of IDGFs and adenosine signaling in cell survival and energy homeostasis".
Briefly, imaginal discs have been productively studied to illuminate global mechanisms of pattern formation and differentiation but much less is known about the intimate cellular mechanisms that regulate survival and/or growth of individual cells in the developing field. Here, Mr. Broz has sought to address that short coming by focusing his studies on two groups of growth regulators that were described in imaginal discs and are highly conserved in mammalian tissues as well, namely the Adenosine Deaminase Growth Factors (ADGFs) and the Imaginal Disc Growth Factors (IDGFs). Mr. Broz has done a nice job of (a) exploring various hypotheses related to mechanism of action and in a quantitative manner (e.g these are not just enhancers of the insulin pathway but totally independent pathways) and (b) determining that IDGFs can affect the adenosine toxicity pathway thus providing evidence that these pathways may cross regulate in vivo. These studies have led to one first author publication and 3 publications as secondary author and have provided important tools and models for future studies into the role of these regulators in cell survival, growth and energy homoeostasis in both mammals and insects.

In my opinion, the thesis is ready for the defense.

Review of the thesis.
Cells in both mammals and insects have evolved mechanisms to maintain cellular homeostasis and viability when faced with various injury or stress conditions. The ADGFs and IDGFs have emerged as possible key players in the suite of stress response pathways. However, mobilization of stress response pathways is often accompanied by pathological levels of inflammation that can be counter productive in todays disease settings. Understanding more about the factors that induce the ADGF and IDGF pathways and the mechanisms whereby they protect cells will advance our ability to understand these stress response pathways and to potentially manipulate them for therapeutic benefit. Thus the pathways being studied here are important, relevant and potentially significant to basic biological homeostasis and potentially to human health.

In the first publication, the author addressed questions surrounding IDGF2 ultimately finding that they function at about the same physiological concentration as in humans (~400mM), that they do not synergize with the insulin pathway but are independent of it. Strikingly, IDGF alone could increase cell viability in minimal media while insulin alone could not and insulin together with IDGF2 did not further increase the IDGF effect. He also showed that IDGF2 can protect cells from the cytotoxic effects of various xenobiotics including dAdo;

thus linking the two pathways and finding that the mechanism of protection is at the level of intracellular processing of dAdo. RNA seq analysis identified a set of genes mobilized in response to IDGF.

These are interesting studies and other studies in the paper raise the question of whether cell in culture with various extracts (e.g. yeast) and or sera are under a chronic state of stress due to things like hi dAdo and Ado?

Gene expression studies show that IDGF2 is expressed in the fat body but rapidly transported to the *Drosophila* nephrocytes (pericardial and garland cells). This raises the interesting question of mechanism of action and what if any effects are cell autonomous and which are non-autonomous and what the signals are.

The second paper makes the case that Idgf3 mutants have hemolymph clotting defects after wounding that include delays in wound repair and that they have increased mortality when exposed to microbial and nematode infection. They nicely address the question of site of synthesis by showing that expression in hemocytes is sufficient to normalize clot formation whereas normal expression in the fat body is also apparently sufficient.

Interestingly he finds that overexpression of IDGF3 leads to increased clot thickness. These interesting studies suggest that they may provide the tools that could be used to devise a screen to identify functionally relevant downstream effectors of the IDGF3 response pathway.

In the third paper, the authors find that susceptibility of cells to Ado stress is highly correlated with the presence of the Ado Kinase (CG11255) and they identify a number of Ado pathway genes whose levels in susceptible and resistant cells are different. In light of some of the cell non-autonomous events in these pathways, these observations raise the question as to which activities may be cell autonomous and which not. In conclusion, they suggest that different cells use different Ado conversion pathways and that the interplay of these may be central to maintaining energy homeostasis in the body.

In the final paper, the authors find that stimulation of the native adenosine receptor (DmAdoR) activates cAMP but not Ca^{++} levels and they identify a series of highly specific agonists and antagonists that can be useful in future screens and studies.

Overall, these studies advance our understanding of the mechanisms of action of the ADGF and IDGF pathways.

In reading the thesis, a couple of questions came to mind that I would like to pose here.

1] The fact that IDGF2's ability to normalize mitochondrial membrane potential in a manner distinct from insulin, raises the question of what the intracellular signaling pathway is. Can the author imagine a screen based on the data emerging here that would allow an unbiased identification of IDGF2 pathway signaling components? Could this screen be used to test the validity of the pathway components identified by transcript analysis? e.g. a similar strategy of vailidating physically identified interactor for bona fide genetic modifier activity was used by Kaltenback et al. PLoS Genet. 2007 May 11;3(5):e82 to achieve an order of magnitude improvement on the identification of modifiers.

2] Given the over and under expression phenotypes seen with IDGF3, could one devise a screen to identify downstream pathway components AND could one use the two screens to determine whether downstream effectors in the IDGF2 vs IDGF3 pathways are the same or different?

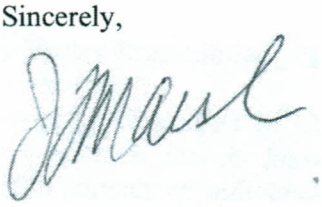
3] One question that seems fundamental to me is to determine which functions of any given IDGF are cell autonomous and which are non-autonomous? How could you design experiments to determine if a given IDGF function was obligate cell autonomous or not?

4] I was intrigued by some of the suggestions for future studies.
a) One was to test the crosstalk between Ado and IDGFs. I am curious what would be the hypothesis to be tested and the experimental design to test that?

5] Finally, how would the candidate speculate that this operates *in vivo* with IDGF proteins being synthesized in one place, transported to another and potentially affecting a tissue in a third place? Are there any obligate cell autonomous functions.

If I can be any further assistance in this matter, please feel free to contact me at jlmarsh@uci.edu.

Sincerely,



J. Lawrence Marsh

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Sincerely,

J. Lawrence Marsh

MASARYK UNIVERSITY
FACULTY OF SCIENCE
DEPARTMENT OF EXPERIMENTAL BIOLOGY

Brno, July 13th 2017

Review of Ph.D. thesis written by Mgr. Václav Brož: Role of IDGFs and adenosine signalling in cell survival and energy homeostasis

Mgr. Václav Brož's Ph.D. thesis describes the role of imaginal disc growth factors (IDGFs) and adenosine deaminase-related growth factors (ADGFs) in regulation of cell survival and energy metabolism in *Drosophila melanogaster*. For this purpose *Drosophila* imaginal disc cell culture Cl.8+ was used and mostly the effects of IDGF2, IDGF3 and extracellular adenosine were tested. Results show importance of IDGFs in immunity including cytoprotection, wound healing, haemolymph clotting and regeneration. Similarly adenosine is involved in responses to stress and infection.

The thesis (167 pages) consists of introduction part, four published articles, concluding remarks and references. The „Introduction" based on 81 references provides the background about IDGFs and ADGFs in *Drosophila* and other insects and also describes the similarities between invertebrates and humans. Aims are clearly described and correspond with obtained results which are briefly summarized before reprints of scientific papers. The chapters „Conclusion and future perspectives" together with „References" are localised at the end of theses; personally I would prefer their replacement before article reprints. The thesis is written in English, the text is understandable, and results are clearly documented and discussed in already published scientific papers.

Mgr. Václav Brož published the results as first author of scientific paper in an international, peer-reviewed journal Scientific Reports (IF = 4.259) which is considered as high quality journal in the fields of molecular biology, entomology or genetics. The thesis contains also other three papers in reputed journals (Journal of Innate Immunity, IF = 3.938; Insect Biochemistry and Molecular Biology, IF = 3.756 and Journal of Neurochemistry, IF = 4.083) where is Václav Brož as a co-author; his individual contribution to team work is stated. The peer-review process in all journal redactions is also a guarantee of results quality.

Comments and questions

- Some summary scheme about multiple effects of studied molecules and e.g. table with insect species where different IDGFs were already described would help the reader with orientation.
- Differences in IDGFs are mention between developmental stages of *Drosophila* when lower amount is present in adults. Is there any evidence of aging influence, e.g. in older adults drop the IDGF amount?
- Presented results of IDGFs are on *Drosophila* cell line, other species have lower amount of genes for chitinase-like proteins, so there are less or no homologs. How we can generalise the results for other insect/invertebrate species?
- Adenosine signalling and metabolism involves lower amount of genes than in mammals, are there also interspecies differences in insects/invertebrates?

Minor correction:

- Page 2: Six ADGF members are mentioned, but there are only five of them (IDGF-A, -A2, -B, -C, -D).
- Haemo- and hemo- should be uniform in the text (e.g. heamocytes, haemolymph).
- Drosophila x *Drosophila* is not uniform, also other Latin names should be all in italic.
- Full Latin names should be written when firstly used (*D. melanogaster*, *C. elegans*….).
- Reference Kwon et al. 2008 is missing in the references.
- Signal pathway impact analysis (SPIA) and Gene set enrichment analysis (GSEA) are not mentioned in the abbreviation list.
- Page 15 – reference „Kawamura et al." and similarly page 160 "Vierstrate et al." – year of publication is missing.

Evaluation:

To produce highly valuable results suitable for publication in reputed scientific journals Mgr. Václav Brož needed large experimental up to date background in molecular biology, genetics and *Drosophila* techniques. Except published articles, he attended foreign stay in USA and is a main author of international conference presentation. Ph.D. thesis is well written and meets the requirements, thus **I suggest accepting the thesis for defence as one part of Ph.D. degree**.

Yours sincerely,

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BIOMEDICAL RESEARCH CENTER
Slovak Academy of Sciences

Review

of the **Václav Brož**' PhD thesis "Role of IDGFs and adenosine signaling in cell survival and energy homeostasis"

Addressing many key developmental, genetic, signaling and disease-prone aspects in biology or medicine is based on the use of well-established model organisms, among which *Drosophila* takes one of the most privileged position. The battery of molecular and genetic tools available in this species allows for unprecedented tasks not seen in other species to be deployed. Presented PhD thesis deals with two widely conserved signaling pathways, imaginal disc growth factor (IDGF) and adenosine, and where mentioned genetic and molecular tools in *Drosophila* were successfully used to obtain very interesting and scientifically valuable data.

Laboratory of professor Žurovec belongs in this field among well known research groups in the european as well as global context. Thus, it is not surprising that there is a continued effort in mining new results in the field, that is clearly demonstrated also in a series of papers which provide the groundwork of this thesis. Adenosine (Ado) is a ubiquitous metabolite and a widespread signaling molecule generated and released from cells following injury or stress. It promotes processes important in both metabolic imbalance and tissue protection. Ado physiological and pathophysiological roles are mediated mainly by the activation of specific G protein-coupled receptors, with its single member found in *Drosophila* genome. On the other hand, IDGFs belongs to chitinase-like protein family, mainly produced by fat body and haemocytes, capable to bind numerous proteins on cell surface, although their functions are not fully understood.

Major part of the dissertation is composed of four important papers, each dealing individually with IDGF2, with IDGF3, with extracellular adenosine, and with the second-messenger stimulation by endogenous Ado receptor (AdoR) under both, *in vivo* as well as *in vitro* experimental conditions.

Author has characterized physiological concentrations and expression of IDGF2 *in vivo* as well as its impact on the viability and transcriptional profile of *Drosophila* cells *in vitro*. He also has shown that IDGF2 is independent of insulin and protects cells from death caused by serum deprivation, toxicity of xenobiotics or high concentrations of extracellular Ado and deoxyadenosine. Transcriptional profiling suggested that such cytoprotection is accompanied by the induction of genes involved in energy metabolism, detoxification and innate immunity. The IDGF2 appears to be an abundant haemolymph component, which is further induced by injury in larval stages. The highest IDGF2 accumulation was found at garland and pericardial nephrocytes supporting its role in organismal defence and detoxification. Obtained data provide evidence that IDGF2 is an important trophic factor promoting cellular and organismal survival.

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In another paper Václav Brož has documented that IDGF3 plays an immune-protective role during infections with entomopathogenic nematodes, when they force their entry into the host via border tissues, thus creating wounds. Whole-genome transcriptional analysis of nematode-infected wildtype and *Idgf3* mutant larvae have shown that, in addition to the regulation of genes related to immunity and wound closure, IDGF3 represses Jak/STAT and Wingless signaling. Further experiments have confirmed that IDGF3 has multiple roles in innate immunity. It serves as an essential component required for the formation of hemolymph clots that seal wounds, and *Idgf3* mutants display an extended developmental delay during wound healing. In all respects, these data significantly contributed to our general knowledge of normal physiological function of the IDGFs or chitinase-like proteins family which are known to be upregulated in several human disorders that affect regenerative and inflammatory processes.

In the course of experiments with *Drosophila* imaginal disc cell line Václav Brož contributed to the paper in which authors have shown that Ado negatively influences viability, changes morphology and mitochondrial polarity of the Cl.8+ cell line via a mechanism exclusively dependent on cellular Ado uptake. High transport of Ado is followed by phosphorylation and ATP production as a part of Ado salvation, which at higher concentrations may interfere with cellular homeostasis. In contrast to Cl.8+, hematopoietic cell line Mbn2, which grows well in high Ado concentration, preferentially utilizes adenosine deaminase as a part of the purine catabolic pathway. Results show that different types of *Drosophila* cell lines use different pathways for Ado conversion and suggest that such differences may be an important part of complex mechanisms maintaining energy homeostasis in the body.

In the last, but not least, of the papers presented in the dissertation, author made contribution to the characterization of the second-messenger stimulation by endogenous *Drosophila* adenosine receptor (DmAdoR) in a neuroblast cell line and examined a number of Ado analogs for their ability to interact with DmAdoR. Authors show that Ado can stimulate cAMP but not calcium levels in *Drosophila* cells, and identified one full and four partial DmAdoR agonists, as well as four antagonists. This study very nicely documented that the employment of the full agonist, 2-chloroadenosine, in flies mimicked *in vivo* the phenotype of *DmAdoR* over-expression, whereas the antagonist, SCH58261, rescued the flies from the lethality caused by *DmAdoR* over-expression.

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, reading author's papers and thesis rises some questions or points of interest which I feel to be addressed, and answered, as follows:

[1] On pages 20-21 and 30 are described data from measurements of mitochondrial membrane potential. It appears clear that data were obtained by flow cytometry, however, I would be interested how quantification of this signal/membrane potential has been made, what has served as reference value (standard) for the mitochondrial membranre potential, and whether these measurements were made in a time-dependent manner ?

[2] In the same section of your thesis you described preparation and subsequent use of recombinant IDGF2 produced by using baculovirus system. Is there available knowledge what are all types of posttranslational modifications of the IDGF2 and whether baculoviral recombinantly produced IDGF2 had all of them ?

[3] In the course of your thesis you mentioned the use anti-IDGF2 and anti-IDGF3 polyclonal antibodies. I did not find specifications of their origin or reference. Could you please specify whether these have been made in your laboratory in the course of this work, or independently as separate task, and where they have been described (incl. specificity) ?

[4] Microarray data on gene expression analysis after nematode infections are very interesting and represent robust piece of scientific work. However, could you explain meaning of GO analysis or data presentation (Fig. 2 p. 66 (page 204 of the journal)) on biological process summary where some ontological categories appears somewhat redundant like defense response to gram-positive bacterium *vs*. defense response to other organism, or humoral immune response *vs*. immune response, or response to external biotic stimulus *vs*. response to biotic stimulus ?

[5] Data on pharmacological effect of the tested AdoR analogs appear very tempting and important. Personally, I do not know how many agonists and/or antagonists of AdoR are available, but there would not be chance to obtain higher number and structurally more diverse adenosine analogs to gain more data so that present study could be expanded to the 3D QSAR and pharmacophore analysis ?

[6] Along these lines, schemes of the potential interactions between agonists or antagonist and A2AReceptor presented in Figures S1 and S2 on pages 156/157 provide some cues to data described in your paper published in J. Neurochem **121** (2012). However, it would not be more appropriate to try to build homology model of *Drosophila* AdoR based on Xu *et al.* (2011) and Lebon *et al.* (2011) coordinates and probe such a 3D model with docking protocols available today to fit tested agonists or antagonists to the AdoR cavity ?

Based on above mentioned facts, generally accepted international requirements for PhD theses, author's contribution of the research data with obtained evidence, experimental methods used, the achievements of Mr. Václav Brož meets 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 excellent grading.

Bratislava 11. 7. 2017

R. Farkaš

RNDr. Robert Farkaš, CSc.
Head of the Laboratory of Developmental Genetics ÚEE BMC SAV