Ph.D. Thesis Review

The thesis of MUDr. Ahmed Ali Chughtai „Evolutionarily conserved mechanisms of gene expression regulation by nuclear receptors" aimed

- *To explore the basic conserved mechanisms that play an intrinsic role in modulating gene expression in metazoans*
- *To identify protein(s) that are responsible for integrating complex environmental, metabolic, structural and stress-related cues and to relay this vital information to the transcriptional machinery*
- *To gain a basic mechanistic understanding of how such protein(s) would function in a cell-specific manner*

(p. 10 of the thesis)

To achieve this, two evolutionarily distant experimental systems were chosen – a primitive *Trichoplax adhaerens* and a more complex *Caenorhabditis elegans* - which were approached by a combination of techniques including bioinformatics, molecular biology, biochemistry and advanced visualization methods of fluorescence and CARS microscopy. Although the methodology used in the thesis is considered appropriate, it seems that the crucial part of the presented results and thus the main message of the thesis diverged significantly from the claimed scope and aims.

Form and elaboration

The thesis is written in English which contains not more than usual number of typos ("*isofrom*" – twice on p. 72; "*that that*" on p. 88; "*panels A image*" on p. 89; "*localize*" and derivatives on p. 21, 79 and 84, but "*localise*" on p. 26 and about forty times more elsewhere in the text,...). Unfortunately, one such a mistake appears already on the title page of the thesis (p. 1) – in the English title of the thesis, the word "regulation" is missing. Identical mistake can also be found on p. 2.

As a reader, I was really missing the decimal classification in the Table of Contents. The absence of short identifiers was probably the cause of almost complete author's suppression of all cross-references within the text. Lack of these cross-references blocked quite efficiently the basic reader's orientation in the thesis. Reading the Results, for example, one repeatedly wants to confer the experimental details with the appropriate parts of the Methods chapter. Similarly, discussing the results, cross-references to specific experimental data are helpful. In this thesis, however, the eighteen pages long (!) Discussion contains just two such cross-references (to Fig. 32 and Fig. 25). The overall readability is further embarrassed by the fact that the three parts of Results were not entitled, just numbered, based on the three different publications, on which the author participated. This Results partition does not functionally participate in the thesis structure, as neither the size nor the data content of Parts I and III are comparable to those of Part II. While Parts I and III are quite short (four pages each, including figures), Part II has almost double the size of both in common.

Minor comments:

- In the list of abbreviations, the explanation of ADRP (p. 5): instead of "*PLIN2*", which is correct but explains nothing, "*Adipose differentiation-related protein, also called Perilipin 2, PLIN2*" would be much more informative for the reader. Also, there is an apparent typo in the

explanation of FLIM (p. 6): instead of "*Fluorescent Lifetime Imaging Microscopy*" should be "*Fluorescence Lifetime...*"

- Third-order headings of Methods and Materials chapter are not listed in Table of Contents, although it is the case for Results and A review of literature. The only exception from this is the first part of M&M chapter (Animal culture, strains, transgenic lines and genome editing), which is "subdivided" into one part, what however does not make a good sense. In contrast, the structure of parts "*Microscopy and Imaging*" and "*Protein synthesis and binding studies*", which are subdivided into three smaller parts each in the text, is not visible in ToC. Again, this effectively prevents the reader's orientation in the thesis.

Science

The most important question of whether the thesis research generated significant new knowledge in a scientific area, has to be answered positively. Yes, there are interesting, potentially important original findings presented in the Results. However, major part of the presented results (Part II of the Results) has a vague relation to the declared objectives of the thesis. The author identified there a nematode protein W01A8.1 as a structural homologue of vertebrate perilipins, localized it onto the surface of lipid droplets in the cytoplasm of embryonic cells of *C. elegans* and characterized the lipid metabolism-related phenotype in the cells following the protein knock-out. This is an admirable piece of work, but it is difficult to trace its connection to the DNA transcription regulation by nuclear receptors.

The author used modern and powerful approaches in the presented experiments. However, these were not always explained well to the reader. Not enough information is provided to allow the reader to understand (and evaluate) the experimental setup. Two prominent examples are FLIM and CARS microscopy approaches:

For fluorescence lifetime imaging microscopy experiment, neither setup nor results are explained in the text. Specifically, it is not clear what time windows were used to separate the GFP signal and autofluorescence, or what was the resulting mutual localization of short-lived fluorescence signal (autofluorescence) and long-lived fluorescence (GFP). This information is completely missing and consequently, it is unclear what was the reason for using FLIM – it is declared that it was to eliminate the autofluorescence, but it is not shown (nor quantified) that the autofluorescence could be eliminated indeed.

For CARS microscopy, the author mentioned in Methods that "*CARS systems allow visualization of lipids of specific categories by tuning into symmetric CH_2 vibrations of specific fat composing molecules (Zumbusch et al., 2013)*". But he doesn't explain what lipid category has been visualized by selection of the Raman shift according to the description in Methods and presented in Results (Fig. 37). What lipid species can we see in Fig. 37? It should be clearly explained what is the relation of lipid containing structures observed by CARS "*at the periphery of the dividing nucleus*" (p. 83) to lipid droplets observed by fluorescence microscopy "*around the cell nucleus*" (p. 82). Are they identical? If yes, what is the evidence for this conclusion? If not, what is the difference between these two? Knowledge concerning the specific lipid content of the CARS-positive structures could provide such information.

Moreover, it is absolutely not clear why a significant part of the Discussion is devoted to the problematics of the possible appearance of lipid droplets within the cell nucleus, while intranuclear lipid droplets are not explicitly mentioned in any part of Results nor highlighted at any of the presented images. Instead, appearance of lipid droplets "*around the nuclei*" is repeatedly pointed out. It has to be accented here that the rim of so called nucleus-vacuole junction, a membrane contact site responsible for the communication between the nuclear envelope and the vacuolar membrane, is a

common place of lipid droplet formation. Therefore, lipid droplets juxtaposed to the cell nucleus are quite frequent and *per se* do not indicate any intranuclear function.

Minor comments:

- On p. 70 the author mentions that "*The 9-cis-RA binding assay showed high affinity binding to GST-TaRXR with a saturation plateau from 5nM to 10nM (Fig. 26C).*" It is unclear how the concentration interval for the plateau was determined, as only the experimental data corresponding to ≤ 2nM and 10nM of 9-*cis*-RA, connected by straight lines, are presented on the respective figure. Where did the information about 5mM concentration come from?
- There are no error bars present in Fig. 26 – does it mean that the experiments were performed only once, or just results of a typical experiment are presented at each panel (Fig. 26A, B, C)? If the latter takes place, it should be stated there how many times the experiment was performed. Similar objection can be raised for the data presented on Fig. 27 (qPCR assay), where "*repeated experiments*" are mentioned in the text of the Figure legend (p. 72), and for Fig. 38C, D, G, H.
- On p. 75, the author suggested "*to rename W01A8.1 as plin-1...*" but did not use this nomenclature in the rest of the text.
- On p. 79, the formulation "*To test the function of W01A8.1, we used RNAi done by germline injection and by feeding*" is a bit too concise and slangy. The reader is further confused by the fact that the abbreviation is used in both meanings "*RNA interference*" and "*small interfering RNA*" throughout the whole text, and is never explained (not even in the List of Abbreviations).
- Fig. 33 – title of the y-axis would be of help.
- Fig. 34 – description of the image presented on the Fig. 34J is inaccurate: "*Large vesicular structures...around the dividing nucleus*" described in Figure legend are identified with lipid droplets, the structures which are by definition not surrounded by the membrane and thus do not have a vesicular character. "*Globular structures*" would be better here, for example.
- Figure 41 is not recalled in the text. Moreover, as the author stated in the Methods, (p. 64): "*the two-channel setup was used to help resolve between spectrally different autofluorescence and GFP fluorescence signals*". Autofluorescence image (Channel 2) should be presented along with GFP signal (Channel 1) in Fig. 41, to show the overlap of the these two. (Contrast of the image presented in Fig. 41I should be improved – the three arrowheads point to almost complete darkness in the printed version of the thesis.)
- The very last part of Results documenting the interaction of F28F8.5 with Mediator complex subunits lacks any image showing the presented data. Is this correct? Even if yes, the information about the experimental error and the method of its assessment is missing in the text.
- The first part of the Discussion (pp. 91, 92) does not mention any aspect of the results presented in the thesis. It is not clear why it is included into Discussion and not to A review of literature.

Conclusions

Concerning the originality, the author's ability to perform research and to achieve scientific results, as well as the author's contribution to the published studies, the thesis satisfies the conditions of a creative scientific work. There is however a significant disproportion between the declared scope of the thesis and the scientific impact of the presented results. In brief, the thesis is mainly about perilipins, and their connection to nuclear receptors mediated regulation of the gene expression seems to be not more than a hypothetical one. I believe that during the oral presentation, the author will be able to explain this disproportion. Only then its contribution can be sufficient to award him with a PhD.

Prague, Nov 8, 2019

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Questions

1. In the introductory part of the thesis, three aims of the work and four goals were demarcated. While there is no doubt that the specific targets (goals) were at least partially met in the thesis, the relation of the presented results to the more general targets (aims) is not easy to follow. Can the author briefly summarize to what extent the aims of work were achieved and how?
2. It is not clear why arbitrary units, and not square micrometers were used to describe changes in the area of lipid-containing structures observed by CARS microscopy (Fig. 38). What was the size of the arbitrary unit? The author mentions including objects with the area of 1-4AU into the analysis. In the case that 1AU would be comparable to one pixel, which is suggested by the presented images (Fig. 37), a question of the noise identification has to be raised. How the noise was eliminated during the analysis? Why CARS was used in this experiment - what is the superiority of CARS microscopy over fluorescence microscopy (staining by lipophilic dyes or by genetically encoded fluorescent proteins, for example) in this particular case?