Ulrike Topf, PhD, DSc
Laboratory of Molecular Basis of Aging and Rejuvenation
Institute of Biochemistry and Biophysics
Polish Academy of Sciences
T: +48 22 591 1319
E: utopf@ibb.waw.pl

Warszawa, 31.08.2022

Review of Aneta Agnieszka Dyczkowska PhD dissertation entitled "Characterizing the roles of ETS-4 transcription factor in fat metabolism"

Aneta Dyczkowska PhD thesis was prepared in the Institute of Bioorganic Chemistry Polish Academy of Sciences in Poznan under the supervision of dr hab. Rafal Ciosk and co-supervised by dr Agnieszka Chabowska-Kita.

The scientific aim of the work was to unravel novel molecular factors that regulate fat metabolism. Such fundamental research is important in the light of an increasing population with obesity and co-occurring diseases. The experimental work was carried out in the nematode *Caenorhabditis elegans*, which is a well-suited model organism to address such kind of research because of established methodology and evolutionary conserved pathways involved in fat metabolism. Specific questions underlying the work were based on previous published and unpublished results of the Ciosk lab. In that work the ribonuclease REGE-1 was identified in a screen for genes necessary to withstand cold stress. Additionally, loss of *rege-1* resulted in decreased accumulation of body fat. The gene *ets-4* was identified as target of REGE-1. Aneta`s work directly relate to these findings and is comprised of three main tasks: First, characterisation of consequences of depletion of REGE-1; Second, determination of functions of ETS-4 target genes; and third, identification of cellular functions that ETS-4 can modulate. Methodologically Aneta`s work is based on various genetic alterations to decipher the *rege-1 – ets-4* signalling pathway. Microscopy and biochemical assay were primary used to assess fat content under different conditions. Aneta`s main findings show that regulation of lipases and sphingolipid metabolism are likely involved in regulating fat metabolism along the *rege-1 – ets-4* signalling pathway.

The experiments to address the first task are essentially repetitions of key findings published by Habacher et al. 2016. The results establish that the decrease of *rege-1* leads to increased expression of *ets-4*, the main target of the ribonuclease REGE-1. Further, knock down of *ets-4* restored the fat accumulation in *rege-1* mutant animals. It was mentioned that *rege-1* mutant

animals display a developmental delay. Did the differences in development have been taken in account during assessing ORO staining? Further in the work many different genes were tested to restore the fat loss phenotype of *rege-1* mutants. Did the genes that rescued the phenotype always rescued the developmental delay? Genes encoding for lipases were tested as potential targets of ETS-4. Expression of *lipl-1* and *-2* were dependent on ETS-4 but did depletion of lipase genes also restored fat accumulation in *rege-1* mutants?

In the second task, Aneta investigated numerous other genes as potential targets of ETS-4. The choice of potential targets that were tested were based on previous results originating from a genetic screen and RNA sequencing results. Mutations in the *mrp-1* gene, encoding for an ABC transporter, were identified as suppressors of fat loss in *rege-1* mutants. Aneta found that expression of *mrp-1* was upregulated in *rege-1* mutants but this regulation was independent of ETS-4. In the discussion part (page 108) Aneta suggested that *mrp-1* can act in parallel to *rege-1 – ets-4* pathway. To test this, Aneta suggested biochemical approach. Is there also a genetic approach feasible to address this question?

Further, Aneta reanalysed published RNAseq data for genes that were upregulated in the absence of *rege-1* but downregulated upon *rege-1*; *ets-4* RNAi. She identified seven such genes including *ets-4* itself, validating the approach. The dependence of the regulation of all six genes (*pept-1, nhx-1, pqm-1, fat-5, sptl-1, sptl-2*) on REGE-1 and ETS-4 was checked by RT-qPCR. Aneta confirmed that the transcript levels of all these 6 genes were controlled by ETS-4 transcription factor. Additionally, *pept-1* RNAi in *rege-1* mutant partially recued the fat loss phenotype but as suggested by Aneta via a different mechanism than regulation of TAG levels.

PQM-1, an antagonist of DAF-16, was analysed. While no rescue of fat accumulation was found in the *rege-1* mutants I do have few questions to this part of the results. In my opinion the data presented do not fully support the conclusion that *daf-16* transcript levels are regulated by ETS-4 (Figure 36A). While there is a non-significant difference compared to WT, the difference in transcript levels was also non-significant compared to *rege-1* mutant. Concerning Figure 37, how the mRNA levels contribute to the change in localization of DAF-16? The change in localization is regulated by alteration in phosphorylation. Does a lack of *rege-1* mimicked starvation, which would cause the relocalization of DAF-16 into the nucleus? Has the localization of PQM-1 been tested?

Within the third task Aneta identified *fat-5*, *sptl-1* and *sptl-2* to be regulated by ETS-4. Aneta concluded that FAT-5 contributes to the regulation of lipid composition in *rege-1* mutants.

While a 20-fold increase in transcript levels is substantial are there any other preliminary data that would support this conclusion?

The most promising finding concerns the genes *sptl-1* and *-2* involved in sphingolipid synthesis pathway. Aneta found that both genes are regulated by ETS-4 transcription factor and that simultaneous depletion of the transcript levels fully restores fat levels in *rege-1* mutants compared to wild type. The increased levels of metabolites of sphingolipid pathway were linked previously to obesity-related conditions. Thus, at the first glance an increase in sphingolipid metabolism in *rege-1* mutants displaying less fat content seems counterintuitive. However, based on published findings, Aneta discussed that increased sphingolipid metabolism can decrease the production of TAG, which was observed in *rege-1* mutants. Aneta`s further observations that mitochondrial function may be impaired in *rege-1* mutants seem of particular interest in regard of enhanced sphingolipid metabolism. Especially the accumulation of ceramide inhibits the function of respiratory chain complexes (doi: 10.3389/fendo.2021.635175). I wonder if such correlation has been considered as mechanism explaining mitochondrial deficiency in *rege-1* mutants.

Finally, I would like to know Aneta`s opinion on the possibility that REGE-1 could be considered as therapeutic target to activate cellular responses that downregulate body fat accumulations keeping in mind the potential adverse effects on mitochondria?

The thesis is composed of a classical structure with introduction, results, discussion and materials and method sections. The introduction was supported by figures and schemes, which were helpful to illustrate complex pathways. In my opinion some things could be improved to help accessibility of the data presented.

- Throughout the figures it was difficult to distinguish when RNAi was used or a mutant strain. Including the allele for the mutants in the figures would make it easier to distinguish;
- Figure 12/ 15 avoid a scheme using an equation;
- Figure 15 omits that forward genetic screens using mutagenesis can also lead to alleles with gain-of-function, a major advantage compared to reverse genetic screens;
- Figure 17 (page 54) was confusing. My assumption is that wild-type worms neither express GFP nor mCherry but rather filter setups that are suitable for visualization of GFP or mCherry were used to detect auto-fluorescence of worms. Only the method section revealed that MRP-1::mCherry is the result of a CRISPR/ Cas9 manipulation.

This should be mentioned in the result section as well;

- The expression pattern of MRP-1::mCherry should be re-evaluated. As far as I can judge from the images presented, Figure 17A (g, h) shows expression in the nerve ring and pharynx muscles and potentially pharyngeal epithelium. Figure 17B (g, h) shows expression in the vulva, uterus and spermatheca. Maybe additional images are more suitable to show expression in vulva muscles;
- When using mutants, it would be helpful to write what kind of mutants that are and consequences for the protein product if any;
- Table 2 (page 65) is insufficient. Despite RNAseq data were published previously it should be mentioned what the number represents, what are the errors of the values and if the change was significant or not.

Despite the above mentioned matters the thesis is overall carefully conducted, experiments are described in a scientific manner and the method section is comprehensive in a way that procedures could be reproduced based on the information.

Aneta`s main findings are original and increase knowledge about an interesting research direction in fat metabolism. Aneta also demonstrated in her work that she is able to integrate and discuss multiple hypotheses in her work, which as a consequence opened new research directions to investigate potential parallel pathways to *rege-1 – ets-4* axis.

The dissertation being the subject of the review fulfills the conditions laid down in the Act of July 20, 2018, The Law on Higher Education and Science (Journal of Laws 2018, item 1668 as amended), the Act of July 3, 2018, Provisions Introducing the Act – The Law on Higher Education and Science (Journal of Laws 2018, item 1669 as amended), and The Rules of Proceeding in the Matter of Awarding the Doctoral Degree in the Institute of Bioorganic Chemistry PAS (Resolution of the Scientific Board of IBCH PAS No. 99/2022/Internet of June 9, 2022) and I recommend that the Scientific Board of the Institute of Bioorganic Chemistry PAS allows it to further steps in PhD defense process.

U. Topf

Ulrike Topf, PhD, DSc