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DISSERTATION REVIEW REPORT

Thesis Title: “Characterizing the roles of ETS-4 transcription factor in fat metabolism”

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The Ph.D. thesis is presented as a monograph and describes the work performed by the candidate throughout the graduation programme. The studies mostly characterised the signalling pathways interacting with the REGE-1 - ETS-4 regulatory axis associated with fat loss in *rege-1* mutants under the overarching research line on the molecular regulation of fat metabolism and obesity, using *C. elegans* as a model. The present Ph.D. programme capitalises on previous research conducted by the supervisor, who characterised *rege-1* and described its association with ETS-4 on the reduction of body fat content.

The dissertation starts with a precise contextualisation of obesity as a major burden in our society, followed by the justification of the use of *C. elegans* as an experimental model to study obesity. Particularly, the candidate instigated the similarities with the human genome and its versatility in genetic analysis studies, followed by a brief introduction to *C. elegans* biology and life cycle; a description of FA metabolism, and signalling pathways involved in lipid metabolism. The aims of the study were subsequently presented and generally included the characterisation of the molecular mechanisms downstream of the REGE-1 – ETS-4 regulatory axis leading to the inhibition of fat accumulation in *C. elegans*. The study firstly described the

phenotypic changes in response to the downregulation of *rege-1* by RNAi in *C. elegans*. Changes in the amount of total body fat were visualized using the Oil red O staining. The second aim was to identify and determine the function of potential ETS-4 target genes after a genetic screening and RNAseq. Thirdly, the aim was to test the effects of ETS-4 on cellular function, through the analysis of genes regulating FA sphingolipid metabolism, glycogen synthesis, and by checking the effect of ETS-4 on cellular respiration. The results were presented and next comprehensively discussed.

The main findings of the study include the characterisation of the following pathways**:**

REGE-1 - ETS-4 axis in fat accumulation

The candidate confirmed the similarities between REGE-1 in C. elegans and human Regnase-1, showing the shared targets and therefore similar functions. It was also established a clear link between upregulation of *ets-4* mRNA and activation of lipases, which promoted catabolism and reduction in body weight.

MRP-1 regulated fat content possibly in parallel to REGE-1 – ETS-4 pathway

Results presented in this section showed a significant influence of sphingolipids and the MRP-1 transporter on the regulation of fat accumulation in nematodes. Although both MRP-1 and SPHK-1 seemed to contribute to the reduction of fat accumulation in *rege-1* mutants, as silencing of their mRNAs allowed for the recovery of the fat content to a small extent. Thus, the modulation of fat accumulation in wild-type animals suggests that MRP-1 and SPHK-1 might function in parallel to the REGE-1 – ETS-4 regulatory axis.

PEPT-1 regulated fat content possibly in parallel to REGE-1 – ETS-4 pathway

The candidate subsequently presented the similarities between *C. elegans* PEPT-1 and human PEPT1 and the anatomical location of the transporter. Subsequently, it was found that high levels of *ets-4* mRNA determined an increase in *pept-1* mRNA, whereas *pept-1* mRNA silencing increased the expression of *pept-2*, and that both PEPT-1 and PEPT-2 could transport di- and tripeptides. Finally, the candidate discussed the importance of further studies investigating whether the lack of functional PEPT-1 enhanced PEPT-2 mediated peptide transport, eventually addressed through the quantification of PEPT-2 protein levels in *pept-1* mutants by western blot analysis. Another observation in this section was that, despite increased LD after RNAi of *pept-1*mRNA in wild-type, the relative TAG levels were lower in comparison to controls. Thus, the overall increase in body fat was explained by increased intracellular pool of amino acids (PEPT-1-mediated peptide transport were increased in the *rege-1* mutants); it was also speculated that PEPT-1-mediated regulation of the body fat content could be achieved through effects on cell acidification in the intestine.

ETS-4 regulated fat accumulation irrespective of DAF-16 and PQM-1

In the next experiment, the candidate explored the crosstalk between the *REGE-1 – ETS-4 pathway* and the factors *DAF-16* and *PQM-1.* Indeed, PQM-1 is a transcription factor regulating the expression of genes responsible for development, growth, and reproduction, whereas DAF-16 was shown to integrate the response from various signaling pathways, such as IIS, AMPK, TOR, germline signaling and JNK signaling pathways, influencing aging, longevity, stress resistance and fat metabolism. Since PQM-1 and DAF-16 were shown to control the transcription of genes involved in major metabolic pathways in *C. elegans*, their effects on the

regulation of body fat content in *rege-1* mutants were investigated. The candidate concluded that the silencing of *daf-16* or *pqm-1* mRNAs in rege-1 mutants did not change their body fat levels compared to *rege-1* mutants, denoting the absence of a crosstalk between the aforementioned genes and *ets-4-rege1* signalling.

SKN-1 regulated fat content possibly in parallel to REGE-1 – ETS-4 pathway

The candidate also checked whether REGE-1 - ETS-4 regulatory axis could indirectly regulate body fat content through the activity of the transcription factor SKN-1. Despite a significant increase in *skn-1* mRNA levels in both wild-type animals exposed to *rege-1* RNAi and *rege-1* mutants and the fact that targeting *skn-1* mRNA by RNAi increased fat accumulation in rege-1 mutants, the recovery of body fat was partial compared to controls. The activity of SKN-1 is known to be tightly regulated by the phosphorylation of particular serine and threonine sites via the p38 / mitogen-activated protein kinase (MAPK) signaling pathway. Given the possible existence of oxidative stress during body fat loss, it was plausible the assumption that some of the phenotypic changes observed in *rege-1* mutants were a result of oxidative stress response and concomitant, but independent, phosphorylation of SKN-1.

The effect of rege-1 depletion on lipid metabolism

The candidate studied the lipid content in *C. elegans*, as lipid composition is known to determine the function and fluidity of cellular membranes, lipids mediate signal transduction (Kahn-Kirby et al., 2004), and are the components of TAG and CE. Indeed, alterations in the lipid composition of the mitochondrial cell membrane can disrupt mitochondrial energy metabolism, cause the development of human diseases such as Alzheimer's and atherosclerosis, and also contribute to obesity. The candidate confirmed the upregulation of *fat-5* in *rege-1* mutants, which suggested that MUFA synthesis could be increased and consequently altered lipid composition. The expression of genes influencing lipid composition was also altered in *rege-1* mutants and some PL were increased while others decreased.

Sphingolipids influence the function of cellular membranes through the formation of lipid rafts, despite their activity and signalling molecules within a wide range of cellular functions. Sphingolipids' excessive production in mammals was stimulated by stress such as oxidative stress, pro-inflammatory cytokines and starvation. The candidate observed the rise in *sphk-1* mRNA levels, and ETS-4 had a significant effect on the mRNA levels of other enzymes related to the ceramide synthesis such as *sptl-1*, *sptl-2*, *hyl-1* and *hyl-2*. Moreover, the inhibition of *sptl-1* or *sptl-2* resulted in partial body fat recovery in *rege-1* mutants, while their simultaneous inhibition caused a complete fat recovery. The observed results suggested that sphingolipid metabolism could be responsible for the regulation of fat accumulation via the REGE-1 - ETS-4 regulatory axis.

Depletion of rege-1 affects energy metabolism

FAs play an important biological role. They act as an energy source and can be stored in the form of TAG in AT. Moreover, they are structural components of the membranes and affect the fluidity of cell membranes and membranes of other organelles, such as mitochondria. PUFAs are responsible for the formation of the PL that form the mitochondrial membranes, such as PC and PE, as well as cardiolipins, which are crucial for optimal mitochondrial function. The transport of electrons through the electron transport chain to the inner mitochondrial

membrane causes polarization of the mitochondrial membrane and generation of an electron potential, which is used for the synthesis of ATP from ADP by oxidative phosphorylation. As a result of oxidative phosphorylation, oxygen is consumed and reduced by protons to form water. Animals with depleted *rege-1* had significantly lower oxygen consumption rate levels compared to controls which may suggest abnormal mitochondrial function. Overall, changes in lipid composition in *rege-1* mutants might have caused structural changes in the mitochondrial membrane, which could influence their ability to produce energy. However, to verify these assumptions, additional experiments would have been required, as lipidomic analyses would potentially determine the exact composition of lipids in the mitochondrial membrane, including cardiolipin.

The candidate concluded that the results demonstrate the complexity of the regulation of body fat content through the REGE-1 - ETS-4 regulatory axis, with the simultaneous activation of various metabolic pathways leading to the reduction of fat accumulation in *rege-1* mutants. Indeed, the candidate found that inhibition of specific pathways partially contributed to fat accumulation in *rege-1* mutants, rather than promoting the full recovery of the phenotype. Nonetheless, the present findings represented important progress in our knowledge of the molecular intricacies regulating fat accumulation in *C. elegans* and humans.

General comments:

The work presented represents a significant contribution to the research field of obesity. The methodology described is scientifically sound and accurately described, which will allow for the reproduction of the experiments. The candidate has demonstrated great knowledge in the area of research, good and varied research techniques, and the ability to analyse the results and their interpretation. Also, the results were clearly described and statistical analysis well conducted. The results were comprehensively discussed, in line with the current state of the art.

Concluding remarks:

The present work highlights very important findings in the field of obesity. I am happy to confirm the assessed thesis fully meets the requirements for doctoral dissertations specified in Art. 187 OF THE ACT of July 20, 2018 LAW ON HIGHER EDUCATION AND SCIENCE (Journal of Laws of 2021, item 478), and I, therefore, suggest the admission of the Ph.D. candidate to further stages of the doctoral dissertation.

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