Report for a Ph.D. Thesis submitted to Faculty of Science, Charles University, Department of Parasiotology

Title: Iron-Sulfur proteins and the role of Iron in the gene expression of *Trichomonas vaginalis*

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Reviewer: Dominique Rasoloson Ph.D.

The distinguished quality of MSc Horváthová's work is reflected in its being published in 2 international peer-reviewed journals. She has made important contributions to the draft genome sequencing of *T. vaginalis* and to the identification of three new members of Hyd machinery in the *T. vaginalis* hydrogenosomes.

MSc Horváthová introduces 4 objectives in her doctoral thesis. (1) Developing a sequence–searching application to screen *in silico T. vaginalis* predicted gene products for putative hydrogenosomal targeting N-terminal sequences (2) characterizing putative [FeFe] hydrogenase maturases from hydrogenosomes of *T. vaginalis* (3) investigating iron-regulated genes of *T. vaginalis* using a transcriptomic approach and (4) investigating iron-dependent changes in *T. vaginalis* hydrogenosomal proteome. The transcriptome and proteome analyses are summarized in a paper in press and in a manuscript in preparation.

Her impressive work tackles different themes in her thesis. The diversity of her thesis subjects, her ability to analyze data from extensive techniques including microarray, EST library sequencing, iTRAQ MS analysis techniques, and her ability to work collaboratively, reflect multifaceted qualities required of a Ph.D candidate.

MSc Horváthová provides a detailed and informative description of FeS clusters, their assembly and the FeS protein biogenesis in anaerobic parasitic protozoa *Giardia intestinalis*, *Entamoeba hystolytica* and *Trichomonas vaginalis*; one of the goals being the identification of FeS proteins and components of FeS cluster assembly machinery that are targeted to the hydrogenosome of *T. vaginalis.* The elaborate introduction emphasizes the overall impact of iron on gene expression in *T. vaginalis*.

New findings and intriguing observations were concluded from her thorough experimental studies (1) Malate preferentially enters the hydrogenosome and serves as a substrate for hydrogenosomal energetic metabolism under +Fe condition. Malate is, however, metabolized in the cytosol via pyruvate to lactate when hydrogenosomal metabolism is ceased under –Fe condition. The switch between hydrogenosomal and cytosolic malate metabolism seems to be important for the ability of trichomonads to quickly adapt to changes in iron availability in their environment. (2) Upregulation of a set of cysteine proteases under iron-restricted growth condition was detected. (3) Three members of the Hyd machinery in the hydrogenosomes were identified and their function in the maturation of H cluster proposed. (4) Differential regulation of individual copies of expanded gene groups, with some copies regulated by iron, while expression of others

was not iron-dependent. Interestingly, the expression of all copies of both Succinyl- coA synthetase subunits appeared to be iron-dependent. (5) Components of the ISC pathway were in iron-restricted cell growth condition, consistent with previously published work. Proteins involved in the energy metabolism and in oxygen detoxification system were, however, under the same growth condition.

I suggest the following questions and recommendations.
The page numbers not being displayed throughout the thesis, the comments and questions are in bold.

- **The main criticism is reserved for the "transcriptome" and/or "proteome" study of the thesis. The analysis investigates the change in gene expression in *T. vaginalis* in response to iron supplementation or iron depletion. However, the study only focuses on iron-regulated genes in cells treated with excess iron and cells grown under iron-depleted conditions.**

  A proper control i.e. "untreated cells" is lacking in this study. Comparing microarray, qPCR and iTRAQ mass spectrometry upregulation and fold change in gene expression between Fe-supplemented cells and Fe-chelated cells does not accurately reflect the ratio/fold change in gene expression without the determination of baseline gene expression in control untreated cells. Comparing change in gene expression in iron supplemented or iron depleted conditions with normal conditions, however, will give the most accurate and acceptable data on the effects of iron supplementation or depletion on gene expression in *T. vaginalis*.

  In the transcriptome paper :
  Section 2.1: Cell cultures
  Cells were grown in iron-rich medium and iron-restricted medium. Untreated control cells, however, is lacking.

  Section 2.4: Microarray sample preparation. Four independent RNA samples from T.vaginalis grown under +Fe and –Fe conditions were compared.
  Untreated cells were not analyzed.

  Section 3.2: Comparative EST analysis. "The upregulation index was calculated as the difference between the RF under +Fe conditions and RF under -Fe conditions".
  When studying upregulation, the data are generally compared with or relative to wild type or untreated cells.

  Section 2.6 qPCR: "The expression levels of each gene **(in cells grown with iron and in cells depleted of iron)** *were normalized to those of the housekeeping gene **β**-tubulin, expression of which is not affected by the availability of iron".* **The obtained data then should be compared to gene expression levels in *T. vaginalis* control "untreated cells" over *β*-tubulin to determine whether genes**

were upregulated in the presence or absence of iron.

The author for example uses "excess iron condition" as "control" in the proteome paper:
Section: Results and Discussion
"To investigate changes of T. vaginalis hydrogenosomal proteome caused by iron limitation, trichomonads were grown in media supplemented with 70um iron chelator. As a control, we used ammonium ferric citrate to a final concentration of 86uM."
Again, to investigate changes caused by iron limitation, protein expression levels should also be determined in *T. vaginalis* cells that are cultured under normal conditions i.e. without iron supplementation or depletion. Iron supplemented cells are not control *per se*, if you are looking for changes in protein expression due to iron depletion.

- *Section 2.1: Final concentration of Fe-NTA 100ul; final concentration of 2-2-dipyridyl 50um.*
  Section 3.1: **Higher concentrations were used** *100mM of Fe-NTA; 50mM of 2-2-dipyridyl.* **Aren't such high concentrations toxic for the cells? Or is this a typographical error?**

- *Section 3.1: Microarray analysis. "To establish the cut-off limit and validate the microarray data, we selected 15 genes for qPCR analyses".* **Please explain how you establish the cut-off limit AND validate the microarray by choosing 15 genes for qPCR analysis? Can you also explain again how you chose the cut-off limit 1.3?**

- *Section 3.1: - line 11,* **correct hydrogenase in bracket to iron hydrogenase.**
  *- line 22, Genes <u>which</u> (***should be <u>whose</u>)** *changes of expression did not reach the cut off limit.*

- *Section 3.4: Conclusions.* **Correct typo "transcriptome".**

- *In Table 1 annotation (transcriptome paper) and Supplementary table S1 (proteome manuscript):* **correct "PFO" to "PFOR".**

- *Section 3.3.2: Hydrogenosomal energetic metabolism. "The gene coding for ME-H was the most highly according to EST analysis (Table S4).*
  Section 3.4: Conclusions. "The highest iron-dependent upregulation was observed for Malic enzyme and PFOR".
  Table S4 shows IRP-like Alpha-actinin with a value of 68, therefore being the highest.

- *Section 3.3.2: Hydrogenosomal energetic metabolism.*
  Please explain the conclusion that the 51-kDa subunit of ME enzyme is being significantly upregulated under +Fe condition according to EST analysis,

however, the upregulation of the 24-kDa subunit is not reaching the cut-off limit? Why do you think 2 subunits of ME are differently upregulated under +Fe condition?

- *Section 3.3.3:* **Comment in Czech to be deleted**.

- **How many of the genes (%) in the EST analysis were found in the microarray group?**

- *"Using microarray analysis and EST library sequencing, we have identified 308 and 336 iron-regulated genes, respectively. According to both methods, approximately half of the genes were in iron-rich conditions, and half were in iron-restricted conditions."*
  How many of the genes were overlapping in both conditions when you compare microarray data and EST analysis result? Can you put it in numbers?

- **In the proteome paper:**
  Figure 1: Ten distinct fractions were obtained **- no 10th fraction in the iron-enriched tube.**

  Figure 2: **- Panels should be labeled A) and B).**
  - Tubulin level panel or other housekeeping gene should be added under the Western Blot panels of cells grown under iron enriched and iron depleted conditions.
  - Quantification of Western blot bands relative to wild type/no drug treatment (not included) will better reflect the upregulation of ME.

  Figure 3: "EM of subcellular fractions".
  - What does each panel represent?
  - What are the particles in the fractions?
  - Size bar is missing.
  - The hydrogenosomes in fraction 7 under iron-depleted condition (if the picture shows hydrogenosomes) appear to be at least 40-fold larger compared to the ones displayed on the iron-enriched panel. Please explain.

  Figure 4: **- Panels should be labeled A) and B).**
  - on Y axis, indicate total activity of the corresponding enzyme.
  - Legends missing for Table 1 and Table 2.

- **Please explain the meaning of "Unique genes were differentially expressed".**

- **Can you briefly elaborate why all copies of both SCS enzyme subunits appeared to be iron-dependent while other enzymes are not?**

Evaluation

MSc Horváthová's thesis is written in a comprehensive straightforward manner. The content and composition of her thesis is of high quality. The contribution of her doctoral dissertation thesis opens up broad research avenues and questions for iron-related topics.

I herewith state that the doctoral thesis of MSc Lenka Horváthová satisfies the conditions of scientific research. This thesis can be accepted and doctoral degree can be awarded after successful defense.

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Baltimore, August 27th 2012