Dr. Pierre Chymkowitch, PhD
Oslo University Hospital
Sognsvannsveien 20,
NO-0372 Oslo, Norway.

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Report on Ewa Leśniewska's PhD dissertation

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Novel layers of tRNA transcription regulation
-From assembly of polymerase RNA III complex to its degradation-

General comments

In this dissertation, PhD candidate Ewa Leśniewska describes the work that she has done under the supervision of Prof. Magdalena Boguta at the Institute of Biochemistry and Biophysics, Warsaw, Poland. This work expands the knowledge on the regulation of the budding yeast RNA polymerase III in normal or stress conditions. The candidate published (as first- or co-author) the majority of the data presented in this thesis in three original research articles. In addition, these findings were discussed in a review article first-authored by the candidate.

These publications reflect the good technical quality of this work and the biological significance of these findings; however more efforts could have been applied to structure this thesis (from introduction to discussion) in order to match the expected quality of a PhD dissertation. Importantly, the introduction is missing key information about topics addressed in the result part (ie: stress response, Maf1, Cancer) and therefore does not emphasize enough on prior art or the importance of studying RNAPIII regulation. Furthermore, beyond the obvious contribution of this thesis to the field of transcriptional regulation, these interesting data could have been highlighted by introducing/discussing how they could be important for the understanding of transcription in higher eukaryotes, stress response, energy homeostasis or human diseases when possible.

Given the scientific achievements presented, I recommend the graduation of the candidate. Nonetheless, given the modest quality of this dissertation, I do not recommend awarding the "honorable mention" at this stage.

Q1: Ewa, Could you please use the time that you will have during your defense to make an oral introduction of your work that would match the quality expected from this PhD dissertation. You will briefly but carefully structure and illustrate the state of the art on each of the main topics that are addressed in your articles and unpublished data: The transcription process (initiation, elongation pausing, read through), the transcriptome, RNAPIII assembly, structure and function, regulation of protein turnover, stress response, relevant human diseases. Given this context, you will explain the
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reasons why these projects were initiated and which important questions you had to answer when you started each of these projects.

Q2: A discussion should not contain any data, as data are commented in the "results" part of a scientific communication. In a discussion, you should rather emphasize on how your findings are important to the field or whether they fit or potentially contradict previous studies. You may also, when possible, speculate on models that your data may suggest. Finally, your work is not the end point of the study of RNAPIII regulation. Therefore, you may also emphasize on the questions that remain to be answered, propose future experiments/hypotheses that invariably arise from any work, and tell us why this would be relevant to perform such experiments. You may keep in mind that going deeper into the understanding of RNAPIII regulation is certainly relevant to human diseases for example.

In this regard, may you please use the time that you will have during your PhD defense to pick a couple of relevant points from your studies that may be discussed based on their potential for future developments.

Editorial

Eukaryotic genes are transcribed by three RNA polymerases (RNAP). In brief, the RNAPI transcribes genes encoding rRNAs, the RNAPII transcribes protein-coding genes and the RNAPIII transcribes genes encoding tRNAs, 5S rRNA and a few non-coding RNAs. Strikingly rRNAs and tRNAs account for 95 % of the total pool of cellular RNA, underscoring that maintaining translational capacity consumes the vast majority of the cell's energy devoted to transcription. As a result, in conditions of energy shortage, i.e. nutrient stress, cells rapidly down regulate the activity of both RNAPI and III as a mean of survival, which triggers dramatic metabolic changes. Furthermore, RNAPIII-transcribed 5S rRNA and tRNAs, which represent 20 % of cellular RNAs, must double in quantity before cell division to ensure proper growth of the daughter cell. These observations suggested that the RNAPIII must be tightly regulated by very specific molecular mechanism in response to intra and extra cellular signals. Research on these mechanisms is conducted in several cellular systems including the budding yeast S. cerevisiae and higher eukaryotes, and lead to the discovery of several conserved pathways involved in the regulation of the RNAPIII transcriptional complex, such as the Torc1 / Maf1 pathway. Additionally, such precise regulation of RNAPIII includes posttranslational modifications of numerous members of this complex by sumoylation, ubiquitination or phosphorylation. Some amino acids targeted by these PTMs were found mutated in human diseases, especially in rare neurodegenerative or neurodevelopmental disorders, which may alter tRNA levels in patient cells. Additionally, tRNA synthesis seems to be deregulated in some cancers, which could be due to high levels of cell division and may contribute or be the consequence of the dramatic energy metabolism changes occurring in cancer cells (i.e. Warburg effect). Altogether these points emphasize on the importance of the research on fundamental mechanisms regulating the

activity of RNAPIII. Finally, a non-canonical function of RNAPIII complex components (i.e. TFIIIB) is shaping the 3D architecture of the chromatin through their insulator function. Understanding the mechanisms regulating chromatin dynamics is one of the current major challenges in molecular biology, as chromatin dynamics may influence cell fate, stress response and carcinogenesis.

The work performed by Ewa Leśniewska falls into this important research axis, as she aimed at discovering fundamental mechanisms regulating RNAPIII components and their involvement in energy homeostasis, metabolism and stress response.

Applied methodology, quality of the results and scientific comments

The quality of the results presented in this dissertation is very good. Nevertheless, I have a few questions regarding the contribution of the candidate to her published articles, the choice of the methods and the interpretation of some of the data.

Q3: What was your contribution to the Genome Res. article? Which questions did you contribute to answer in this paper?

Q4: You state that you used CRAC because of the "limited spatial resolution of ChIP-seq".
Could you please explain this? Indeed it sounds inappropriate to compare two methods that do not address the same questions.

Q5: The main conclusion of this paper is that RNAPIII distribution on tRNA genes is uneven. It is an important finding. However, your RNAPIII profile is very similar to the classical profile observed at RNAPII-transcribed genes: a strong peak at the TSS and a weaker one at the TTS.
Why do you think that these two peaks are due to a slow down at A and B boxes? Are there any other examples of such mechanism were a polymerase slows down due to steric limitations?

Q6: Why is it important to study the binding of Rbs1 to RNAs?

Q7: You have pulled down very few RNAs with Rbs1, including some of the highest expressed mRNAs: *PMA1*and RP genes. This allows challenging the specificity of this experiment.
Which extra experiments could be performed to make sure that these RNA species do not constitute the background of your experiment?

Q8: What are the prospects for this project? What are the questions that remain to be answered and the corresponding experiments to be performed?

Q9: What is known about the consequence of MPA on transcription? Does it trigger a specific stress response?

Q10: May you describe the mechanism of polymerase pausing? Does this occur in Saccharomyces cerevisiae? Has it been observed in the case of the RNAPIII?

Q11: TFIIIC ChIP is higher in stress conditions.
Could TFIIIC act as a bookmark when cells undergo stress, allowing transcription to resume faster when conditions become better?

Q12: C160 degradation upon stress.
In your previous MCB paper the *rpc128-1007* mutation was proposed to alter the assembly of RNAPIII. However, one could also conclude that due to lower steady state levels of other RNAPIII subunits in this mutant, this mutation could only alter protein stability/expression rather than complex assembly. This is what you seem to show in this BBA paper, where you actually demonstrate that C160 protein levels are lower in the *rpc128-1007* mutant.
Your BBA data thus seem to contradict the conclusions from the previous MCB paper.
Is the *rpc128-1007* mutation leading to RNAPIII subunits degradation or RNAPIII assembly defect? Or both?

Q13: You suggest that ubiquitination of C160 occurs downstream of RNAPIII inhibition and release from the chromatin. However, a recent study by the group of Tony Hunter indicates that Sumo-dependent ubiquitination of C160 occurs on the DNA before the non-functional polymerase is released.
Do you have any evidence suggesting that ubiquitination occurs in the nucleoplasm and not on the chromatin?

Q14: Sumoylation of C53 is a prerequisite to the degradation of C160 by the proteasome.
Do you think that sumoylation is involved in the degradation of C160 in you experimental settings, where you use MPA or YPGly to stress cells?

Q15: C160 degradation upon MPA or 6AU treatment
Did you check whether these treatments could decrease transcription of C160, C82 or C53 genes?

Q16: Page 102, "the levels of C53 and Maf1 phosphorylation were also not affected".
Why did you test this?

How can you be sure that these moderate mobility shifts of C53 and Maf1 are for sure phosphorylated forms of these proteins?

Q17: C53, C82 and AC40 protein levels degrease in YPGly
Do you think that it is also due to ubiquitination? Please explain the reason for showing Fig 48B: Why did you do a phos-tag gel to address C82 and C53 protein levels?

Q18: In a broader context, Willis and colleagues just published that maf1 KO mice display elevated tRNA synthesis in all organs, and that such elevated polIII activity is the reason for why these mice have an elevated demand in energy. However, the pool of mature tRNAs remains largely unaffected even if de novo synthesis is higher than in WT mice. They propose that extra tRNAs produced in maf1 KO cells are degraded by a "utile RNA cycle". In this cycle, which involves significant metabolic changes, the extra pool of tRNAs is processed to produce the nucleotides that are necessary to fulfill the high demand of RNAPIII.
Considering these data, do you think that the transcriptional phenotype that you observed in MPA-treated cells could be reverted by maf1 deletion? Do you think that maf1 deletion could prevent C160 degradation by the proteasome?
Regulation of C160 degradation by the proteasome could be a general mechanism allowing cells to adapt tRNA synthesis to metabolic changes and therefore modulate energy consumption and/or expenditure?

Conclusion:

Given the scientific achievements presented, this work fulfills the requirements of a PhD. I therefore recommend the graduation of the candidate. Nonetheless, given the modest quality of this manuscript, I do not recommend awarding the "honorable mention" at this stage.

Pierre Chymkowitch