DIOSCURI
CENTRE FOR RNA-PROTEIN INTERACTIONS
IN HUMAN HEALTH AND DISEASE

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To: Scientific Council of the Discipline of Biological Sciences of Adam Mickiewicz University

Review of mgr Daria Niewiadomska PhD thesis

'The role of cis regulatory elements in mutant mRNA of FMR1 gene containing expanded CGG repeats in R loop formation and regulation of noncanonical translation of pathogenic protein'

Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability. It is caused by an expanded CGG repeat mutation in the FMR1 gene, which is located on the X chromosome. The mutation, present in the 5'UTR of the FMR1 mRNA, causes the gene to produce less or no FMRP protein, which is essential for normal brain development. While full mutation expansion leads to FXS, premutation with a smaller number of repeats can lead to Fragile X-associated tremor/ataxia syndrome (FXTAS). This neurodegenerative disorder is characterized by tremor, ataxia, cognitive decline, and behavioural problems. Both FXS and FXTAS are lifelong conditions and there are nor cures for either condition. Another syndrome, associated with CGG premutation in the FMR1 gene is called Fragile X-associated premature ovarian insufficiency (FXPOI), but it has largely been omitted from the presented PhD thesis.

Many diseases, including FXS and FXTAS, are caused by misregulation of gene expression. It is therefore important to study and understand all the processes that contribute to normal and abnormal transcription, RNA processing and translation. This thesis focuses on two major phenomena that are involved in the regulation of FMR1 gene expression:

1. R-loops: These are RNA-DNA hybrids that can form when RNA polymerase transcribes the FMR1 gene. R-loops can alter transcription and translation, and they may play an important role in the development of FXS and FXTAS.
2. Repeat-associated non-AUG initiated translation (RAN): This is a process by which the FMR1 gene can be translated even though it does not have an AUG start codon. RAN can lead to the production of toxic proteins that contribute to the pathogenesis.

R-loops are nucleic acid structures that form when RNA polymerase transcribes a gene, and the RNA transcript remains bound to the DNA template strand. They consist of an RNA-DNA hybrid and a single-stranded DNA segment that has been displaced, and they interact with numerous protein factors that serve to stabilize or resolve them. R-loops can occur in all cells, but they are more common in cells that are actively transcribing genes. They also have a higher propensity to form on sequences with high GC content, such as the CGG repeats in the FMR1 gene. R-loops can interfere with transcription and translation, and they can also damage DNA. They are therefore thought to play a role in the development of FXS and FXTAS. Of note, there are several drugs and other therapies that are being developed to target R-loops. These treatments have the potential to address a range of diseases in which R-loops play a role in the underlying molecular pathology.

During RAN process proteins can be synthesized from mRNAs that using non-AUG start codon. RAN is thought to play a role in the development of several diseases, including those with trinucleotide repeat expansion. In RAN, the ribosome scans the RNA transcript for a non-AUG start codon. The ribosome then binds to this start codon and begins translation. The proteins that are produced by CGG repeat non-AUG initiated translation are often toxic, such as FMRPpolyG. The selection of the non-AUG start codons and the strength of the translation initiation from these sites are not fully understood. Therefore, it is important to study the primary and secondary structures of the RNA transcript that influence this phenomenon.

The PhD thesis analysed mechanisms that are involved in the regulation of FMR1 gene expression. The methods used were mainly *in vitro* synthesized RNAs and cell culture. The main conclusions of the thesis are:

1. FMR1 CGG repeats form R-loops *in vitro* and in cells.
2. Antisense oligonucleotides against CGG can reactivate FMR1 transcription, most likely by relieving the block of R-loops.
3. The primary sequence and secondary structure have a significant impact on the translation efficiency of FMRPpolyG proteins initiating from non-AUG start codons.

The PhD thesis has a well-structured canonical format. The results section is divided into two main parts – R-loops and RAN. All figures and figure legends are well-presented. I would like to highlight the comprehensive introduction and discussion sections. The candidate clearly demonstrated her knowledge and understanding of the field. She has also made a significant contribution to the field through her research, which is evidenced by high profile publications.

Here are some more detailed comments regarding the PhD thesis:

1. The FXPOI (Fragile X premutation onset of instability) should be mentioned and discussed in the context of R-loops as well as RAN translation. Do we observe comparable molecular phenotypes in both FTAS and FXPOI? What governs the specificity of their physiological phenotypes?
2. Throughout the part of the thesis that deals with R-loops, biochemical methods have been used to detect them. Why was there no attempt to utilize anti-R-loop antibody, such as S9.6, to detect and confirm the presence of these structures in vitro and in cells?
3. The R-loop smear should have been quantified and presented alongside quantification of the produced RNA. Currently, we can only visually assess whether the R-loop smear intensity is stronger or weaker.
4. The experiments presented in Fig. 21 have been analysed at a single time point. While R-loop formation is a dynamic process and compensatory mechanisms could be taking place in the RNAi cells, one should analyse the FMR1 mRNA with expanded CGG repeats in a time-dependent manner.
5. The upregulation of FMR1 mRNA was significant upon ASO-CCG treatment in FXTAS cells (Fig. 22). Why did the author not include western blot analysis on these samples? Was there no change in the FMRP protein levels in a similar manner to the one observed in FXS (Fig. 24)?
6. Are there any single nucleotide polymorphisms (SNPs) in the general population or in patients with FXS or FXTAS in the vicinity of the RAN translation near-cognate start codons? If yes, perhaps these SNPs could regulate the amount of FMRpolyG or other RAN translation can contribute to pathological processes.

7. From the medical perspective, would it not be better to provide replacement therapy with FMRP coding mRNA or AAV plasmid? Are such approaches being attempted or planned by academia or the medical industry?
8. Is it possible that the newly discovered function of the FMRP protein in the nonsense-mediated decay (NMD) pathway (Kurosaki et al., 2021, Nature Cell Biology) could interfere with the production of FMR1 mRNA or FMRP protein?

The doctoral dissertation submitted for review meets the conditions specified in the Law of July 20, 2018. - Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended), Law of July 3, 2018. Introductory Provisions of the Law - Law on Higher Education and Science (Journal of Laws of 2018, item 1669, as amended) and in the procedure for granting the degree of doctor by Adam Mickiewicz University, and I request the Scientific Council of the Discipline of Biological Sciences to admit mgr Daria Niewiadomska to the further stages of the proceedings for granting the degree of doctor and to award this dissertation. Furthermore, due to the exceptionally high level of the achieved results, I am requesting that the PhD thesis be recognized with a distinction.

With best regards,

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